Aquaculture

Dietary supplementation with Aloe vera induces hepatic steatosis and oxidative stress together with a disruption of cellular signaling pathways and lipid metabolism related genes' expression in gilthead sea bream (Sparus aurata)

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May. 26th, 2022

Dear Editor-in-Chief of Aquaculture Journal

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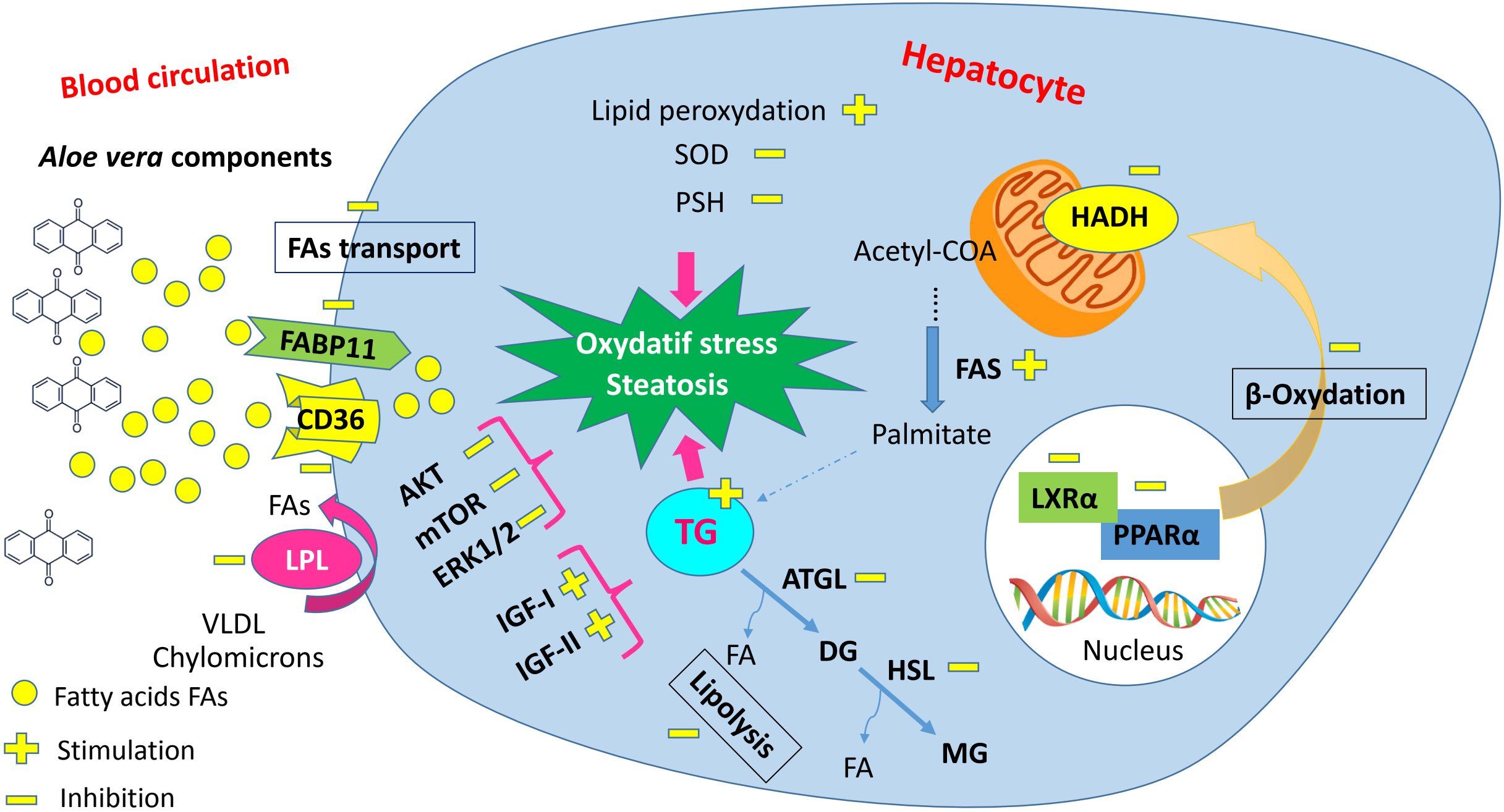
Highlights

- The effects of dietary *Aloe vera* (AV) powder were studied in Gilthead sea bream.
- HPLC-DAD-ESI-MS analysis revealed the presence of anthraquinones in (AV) powder
- Dietary AV powder reduced genes' expression involved in lipolysis and beta-oxidation
- Fatty acid synthase was significantly up regulated by dietary AV powder.
- Dietary AV induced oxidative stress and reduced cell survival pathways-related genes expression



Graphical abstract

Dietary supplementation with *Aloe vera* induces hepatic steatosis and oxidative stress together with a disruption of cellular signaling pathways and lipid metabolism related genes' expression in gilthead sea bream (*Sparus aurata*)

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Abstract

This study aimed to assess the effects of dietary increasing concentrations of *Aloe vera* (AV) powder of 0.5%, 2.5% and 5% on the growth performance, hepatic oxidative status, histology, and lipid metabolism and cellular signaling pathways-related genes' expression in gilthead sea bream (*Sparus aurata*). The preliminary phytochemical analysis revealed the richness of the dried AV extract on total phenol content, total flavonoid content, and condensed tannins when compared to the lyophilized sample. The dried extract showed a good DPPH-radical scavenging activity and its profiling by HPLC-DAD-ESI-MS revealed the presence of anthraquinones namely aloin A, aloin B and their hydroxyl (7-hydroxyaloin A and 7-hydroxyaloin B) and methyl-hydroxy (8-O-methyl-7-hydroxyaloin A and 8-O-methyl-7-hydroxyaloin B) derivatives as well as aloeresin A and B. The AV supplementation in fish diet did not affect growth performance (WG, WGR, and SGR) and feed utilization (FI, FCR, FER), and HSI indexes. However, the hepatic insulin-like growth factors (IGF-I and II) levels were significantly enhanced. Genes' expression levels of enzymes or transcription factors involved in lipolysis (*lpl*, *hsl*, and *atgl*), beta-oxidation (*pparα*, *hadh*), fatty acid transporters (*cd36, fabp11*) and *lxrα* were significantly down-regulated by the two high concentrations of AV powder. In contrast, fatty acid synthase (*fas*), a key gene of lipogenesis was significantly up regulated by dietary AV 5% powder supplementation. The induction of *fas* together with the down-regulation of peroxisome proliferator-activated receptor (*pparα*) and hydroxyacyl-coenzyme A dehydrogenase (*hadh*) could explain the lipid accumulation resulting in hepatic steatosis, which was confirmed by histological analysis, since the diets at the two higher concentrations (AV 2.5% and AV 5%) induced a significant increase in the number and diameter of hepatic lipid vacuoles in a dose dependent manner. Moreover, the mRNA levels of protein kinase B named (*akt*), mammalian target of rapamycin (*mtor*) and extracellular regulated kinase (*erk1/2*) involved in cell survival and proliferation were decreased by all AV powder supplemented diets. AV 5% increased catalase and glutathione S transferase activities suggesting a cellular strategy to fight against reactive oxygen species (ROS) accumulation. In conclusion, dietary supplementation with AV 0.5% is recommended for gilthead sea bream feed formulation, as it stimulates the *igf-i* expression. However, higher levels of AV should be avoided as they might cause lipid metabolism disruption, oxidative stress and liver steatosis.

Key words: *Aloe vera*, *Sparus aurata*, lipid metabolism, oxidative stress, gene expression.

1. Introduction

During the last decades, interest in using medicinal plants as feed additives in aquaculture has escalated due to their richness in bioactive ingredients with health beneficial effects. They are incorporated in farmed fish diet as crude powder/extracts, active compounds or in association with a probiotic (Awad et al., 2017). The use of plant-enriched diets has been shown to improve growth performance, antioxidant activities and immune system in fish (Yousefi et al., 2020; Zemheri-Navruz et al., 2020). However, some plants may contain anti-nutritional compounds or toxic metabolites. Therefore, careful phytochemical examination of the plant and dosage is required before their use as feed additives in aquaculture (Reverter et al., 2020). Among medicinal plants, *Aloe vera* (AV) has received particular attention owing to its antioxidant, anti-obesity, hypo-glycemic, hepato-protective, and immuno-modulatory properties (Maan et al., 2018; Kumar et al., 2019). Earlier compositional investigations showed that AV contained essential minerals, vitamins, saponins, polysaccharides, phenolic compounds including anthraquinones/anthrones (aloe-emodin, anthranol, aloin A and B, emodin, etc.), organic acids, amino acids, and lipids, which make it an essential ingredient in different cosmetic, pharmaceutic and agrifood products (Radha et al., 2015; Mann et al., 2018). Phytosterols from AV were found to induce the overexpression of genes involved in fatty acid transport (*acsl1*), fatty acid oxidation (*acaa1a*, *acox1*, *cpt1a*, *cpt2*, *cyp4a10*, and *cyp4a14*), and retinoid X receptor (*rxr*) in obese mice (Pothuraju et al., 2015). However, the AV anthraquinones were shown to be responsible for the toxicity in primary rat hepatocytes and may be the main constituent responsible for liver injury (Liu et al., 2020). Of all the components of AV, aloin is the most toxic anthraquinone and it appears in two forms: aloin A and B (Kaparakou et al., 2021). Despite the evidence on the differential effects of AV components, there are limited data

on the potential effect of this plant on the transcription network that regulates the hepatic lipid metabolism in fish, specifically, in gilthead sea bream.

Lipid metabolism in fish is a complex phenomenon accrued in liver and other organs such as adipose tissue and muscle; it is widely regulated via the interaction between hormones, transcription factors, growth factors and the involvement of many enzymes in the lipogenic and lipolytic pathways (Bullón-Vela et al., 2018). Transcription factors including liver X receptor (*lxrα*) and peroxisome proliferator-activated receptors (*pparα*) are of particular importance in controlling lipid metabolism in fish (Bou et al., 2014; Wei et al., 2017). The former *lxr* factor is involved in cholesterol metabolism (Ayisi et al., 2018), while the latter *pparα* is implicated in the β-oxidation of fatty acid (FAs) originally produced though lipoprotein lipase-mediated hydrolysis of triglycerides and very low-density lipoproteins (Kidani et al., 2012; Weil et al., 2013). Therefore, a disturbance in that process resulted in a reduced capacity of utilization of free fatty acids (FFA) energy, and consequently, development of steatosis (Bullón-Vela et al., 2018). In general, metabolic disturbance are caused by the overproduction of reactive oxygen species (ROS) leading to oxidative stress (Bullón-Vela et al., 2018; Kim et al, 2020).

Gilthead sea bream (*Sparus aurata*) is considered one of the most important fish species farmed in the Mediterranean area including Tunisia using intensive farming system. Its production increases annually, and is estimated nearly 64,000 tones (Konstantinidis et al., 2021). In capture fisheries, growth performance of Sparus aurata is conditioned by numerous factors including temperature, feed composition and feeding strategies, among others (Mongile et al., 2014). To improve growth performance in farmed fish, feeding strategies based on replacement of fishmeal (FM) and fish oil (FO) have been reported (Xu et al., 2019). Furthermore, other feeding strategies including the supplementation with minerals (Dominguez

et al., 2017), FAs (Turkmen et al., 2020), vitamins (Lanyau-Domínguez et al., 2021), and plant extracts (Nhu et al., 2019) have been successfully assayed. Feeding these functional diets resulted in improved growth with a concomitant enhanced immunity and disease responses as well as increased appetite (Citarasu, 2010).

Prompted by these antecedents and considering the multiple properties of AV, we investigated the chemical composition of AV powder and assessed its potential effects on the growth performance of gilthead sea bream. A special attention was paid for the first time to the hepatic lipid metabolism and cellular signaling pathways related genes' expression, as well as antioxidant defense and liver histology under standard rearing conditions.

2. Material and methods

2.1. Plant sample

Leaves of AV were collected in the region of Teboulba (East-Central Tunisia; Latitude 35°64'86" (N); Longitude 10°97'48" (E)). After washing with tap water, they were cut off from their base and placed vertically for 2-4 h to remove the latex liquid containing anthraquinones (Zanuzzo et al., 2017). Thereafter, they were cut into small pieces and divided into 2 batches. The first batch was dried at 50°C in a forced-oven air for 12 h and powdered using a laboratory mill. The second batch was lyophilized for 72 h using a Christ Alpha 2-4 freeze drier (Osterode, Germany). The dried and lyophilized powder samples were stored at -20°C until processed.

2.2. Preparation of extract

The powdered raw material was extracted using methanol (1:20, w/v) for 24 h at room temperature under constant shaking using an orbital shaker. The combined supernatants were filtered through Wattman filter paper (0.22 µm) and concentrated under reduced pressure in a

Heidolph rotary evaporator (Schwabach, germany). The resulting dried methanol extract was subsequently assayed for its phytochemical analyses and biological activities.

2.3. Phytochemical analyses

All phytochemical measurements were performed in triplicate for each analysis.

2.3.1. Determination of total phenolic content (TPC)

The TPC was determined using the Folin-Ciocalteu (FC) reagent as described by Singleton & Rossi (1965). Briefly, a 500 µL aliquot of sample extract dissolved in methanol or the standard gallic acid (GA) was mixed with 2.5 mL of 10-fold diluted FC reagent. After 4 min, 2 mL of 7.5% NaCO3 was added and the mixture was left to stand for 2 h in the dark at room temperature. The absorbance was then measured at 760 nm and the TPC were expressed as mg of gallic acid equivalents per gram extract (mg GAE/g extract).

2.3.2. Determination of total flavonoid content (TFC)

For the determination of TFC, the colorimetric method with AlCl3 was used with modifications (Dehpour et al., 2009). Briefly, a 500 µL aliquot of sample extract or the standard quercetin (Q) was mixed with 1.5 mL methanol, 100 µL 10% AlCl3, 100 µL 1 M potassium acetate, and 2.8 mL distilled water. After 30 min incubation at room temperature, the absorbance was measured at 415 nm and the results were expressed as mg quercetin equivalents per gram extract (mg QE/g extract).

2.3.3. Determination of condensed tannins (CT)

The CT was estimated using the vanillin method as described by Broadhurst and Jons (1978). A 500 µL aliquot of sample extract or the standard catechin (C) was mixed with 3 mL 4% vanillin (in methanol) and 1.5 mL concentrated HCl. After 2 h incubation at room temperature,

151 the absorbance was measured at 500 nm and the results were expressed as µg catechin
152 equivalents per gram extract (µg CE/g extract).

153 **2.3.4. Characterization of phenolic compounds by HPLC-DAD-ESI-MS**

154 The HPLC-DAD-ESI-MS analysis was performed on an Agilent 1100 series HPLC systems
155 equipped with a photodiode array detector (PDA), a triple quadrupole mass spectrometer type
156 Micromass Autospec UltimaPt (Kelso, UK) and an electrospray source (ESI) ion source
157 operating in negative mode. The column was a C18 reversed-phase Superspher®100 (12.5 cm
158 × 2 mm id; 4 µm, Agilent Technologies, Rising Sun, MD) and its temperature was maintained
159 at 45°C. The mobile phase consisted of a combination of A (0.1% acetic acid in water) and B
160 (acetonitrile) with a flow rate of 0.25 mL/min. The solvent gradient consisted of a multi-step
161 linear gradient: from 0 to 2% B in 5 min, 88% B at 75 min, and decreasing to 2% B in 90 min.
162 The Uv-Vis spectra were recorded from 200 to 800 nm, and ions in the m/z range of 100-1000
163 were detected using a scan time of 1 s. The ESI source was conducted under the following
164 operating conditions: capillary voltage, 3.2 kV; cone voltage, 115 V; probe temperature, 350°
165 C and ion source temperature, 110° C (Mejri et al., 2018). Data acquisition and analysis were
166 performed with a Masslynx software version 4.0. Because of the lack of authentic standards,
167 the tentative identification of compounds was carried out comparison of their UV and mass
168 spectra, as well as their fragmentation pattern with literature data (Koyama et al., 2009; Fanali
169 et al., 2016; El Sayed et al., 2016)

170 **2.4. DPPH (2, 2'-diphenyl-1-picrylhydrazyl) radical scavenging activity**

171 The DPPH-free radical scavenging activity of the methanol extract of AV was determined
172 according to the method of Binsan et al. (2008). One milliliter sample extract at various
173 concentrations (from 0.01 to 2 mg mL-1) was added to 2 mL of 0.1 mM DPPH methanolic

solution. The mixture was Vortexed (Stuart SA8, Thermo Fisher Scientific Inc., Bordeaux, France) vigorously and allowed to stand for 30 min in the dark. Thereafter, the absorbance was measured at 517 nm against the control blank containing methanol and performed in triplicate. The percent inhibition of DPPH-free radicals was calculated as follows:

$$\% \text{ Inhibition} = [(A \text{ blank} - A \text{ sample})/A \text{ sample}] \times 100$$

Where A blank is the absorbance of the control and Asample is the absorbance of extract sample.

The DPPH-radical scavenging activities were expressed as EC50 that represents the sample concentration required to reduce the initial DPPH concentration by 50%.

2.5. *In vivo* experiments

2.5.1. Preparation of experimental diets

Four experimental diets were prepared; a control diet without AV powder and three more diets were supplemented with graded levels of AV powder. The composition of the commercial diet is shown in table 1 [crude protein (45.1%), fat (19.1%), ash (6.7%) and cellulose (3.5%)] (Skretting, Spain, 4 mm). This diet was ground into powder; thereafter, the crude powder corresponding to 0.5 %, 2.5% and 5% of AV was weighed and they were blended together manually about 10-15 min. Then, distilled water was added bite by bite until a stiff dough resulted as required. The control diet underwent the same steps but only with water. The paste of each diet was then separately passed through a mincer with 4 mm die resulting in strands, which were gently broken into fresh pellets, air-dried at ambient temperature for 3 days (Gabriel et al., 2015; Amri et al., 2020). The diet was prepared twice a week.

2.5.2. Fish acclimatization and experimental design

A total of 144 healthy gilthead sea bream fish were obtained from the National Institute of marine Sciences and Technologies of Monastir (INSTM, Monastir-Tunisia). Fish were held in 12 tanks (1000 L, 12 fish/tank) and initially fed with a commercial diet (Skretting, Spain, 4mm) twice a day (9:00 AM and 03:00 PM) during one week for acclimatization. The physicochemical proprieties of seawater were monitored every 10 days during the trial. Temperature, 22±1°C; salinity, 37±2 mg/l; oxygen, (>=3.5 mg/l) and photoperiod (12 h light/12 h dark). The open circuit system (flow-through) was adopted for the seawater continuous changing at the flow rate of 100%/h and with continuous aeration.

After the adaptation of fish to the experimental system they were anesthetized with 2-phenoxyethanol (0.2 mL L-1; Sigma). For each fish, the initial body weight and length were measured to calculate the biometrics parameters. Four groups of fish were installed and received the experimental diets as following: group 1 fed with control diet (without AV), group 2 fed with A.V 0.5% supplemented diet (5 g/kg feed), group 3 received dietary AV 2.5% (25 g/kg feed) and finally group 4 dieted with A.V 5% (50 g/kg feed). The experiments were done in triplicate (36 fish/group). Each fish group was fed with the corresponding diet by hand for 60 days, 6 days a week, 2 times a day (9:00 AM and 03:00 PM) until satiation.

2.5.3. Sampling

After 8 weeks feeding period, fish were fasted 24 h before sampling to avoid regurgitation of food. Thereafter, fish were euthanized by a blow to the head under anesthesia by phenoxyethanol (0.2 ml/l) diluted in seawater (Sánchez-Nuño et al., 2018). The fish were weighted, measured and dissected, and liver was gently rinsed in phosphate buffered saline (PBS), blotted dry and then divided into 3 parts. The first part immediately placed in RNA later

(25 mM sodium , pH 5,2; 1:10 w/v) left at 4°C overnight and subsequently stored at -80°C until gene expression analysis (6 samples/group). The second part of the liver was fixed in phosphate buffered formalin 10%, pH =7.4 for 24 h and dehydrated in ethanol (70%) for histological examination (9 samples/group) and the last part of the liver was stored at -80°C for biochemical analysis (10 samples/group).

2.5.4. Fish growth, survival and feed utilization performance

The individual body weight and length were measured at the beginning and at the end of the AV feeding. Growth parameters and survival rate were measured according to Amri et al. (2020) and as follows:

Weight gain; WG (g) = final mean weight- initial mean weight.

Weight gain rate; WGR (%): weight gain rate =100*(final total weight (g) – initial total weight (g) / initial total weight (g).

Specific growth rate; SGR (%/day-1): Specific growth rate = 100* (Ln final individual weight (g) – Ln initial individual weight / numbers of days).

Condition factor; CF (%) = 100*(final individual weight (g) / final individual length3.

Hepatosomatic index; HSI (%) = 100* liver weight (g) of final individual fish / final individual weight (g).

Feed intake; FI (g/fish): dry feed intake /number of fish.

Feed efficiency ratio; FER (g/g): = WG (g)/ FI (g).

Survival rate (%): 100* (final number of fish) / (initial number of fish)

2.6. Molecular analysis

2.6.1. RNA extraction and cDNA synthesis

As previously described by Balbuena-Pecino et al. (2019), total RNA was extracted from 40 mg of liver with 1 ml of Tri Reagent solution following the manufacturer's recommendations (Applied Biosystem, Alcobendas, Spain). A NanoDrop 2000 (Thermo scientific, Alcobendas, Spain) was used to determine total concentration and purity. Analysis by 1% agarose gel electrophoresis stained with SYBR- safe DNA gel stain (Life Technologies, Alcobendas, Spain) was used to check the integrity of samples. To remove all genomic DNA, total RNA (1000 ng) were treated with DNase I (Life Technologies).Thereafter, RNA was reverse transcribed with the transcriptor first strand cDNA synthesis kit (Roche, Sant Cugat del Valles, Spain).

2.6.2. Real time quantitative PCR (qPCR) analyses

The mRNA transcript levels analysis of the target genes plus two reference genes were examined in CFX 384™ real time system (Bio Rad , El prat de Lobregat , Spain) following the procedures previously described by Balbuena-Pecino et al. (2019). All the analysis were performed in triplicate wells using 384 well plates with 2.5 µl of iTaq universal SYBR Green supermix (Bio-Rad), 250 nM final concentration of forward and reverse primers (Table 2) and 1 µl of diluted cDNA for each sample, in a final volume of 5 µl. The expression level of each analyzed target gene was calculated using the Pfaffl method (Pfaffl, 2001) relative to the geometric mean of the two reference genes (rps18) and (ef1a) as they were both constitutively expressed and not affected by the treatments. Both, relative expression calculations and reference genes stability determined by the geNorm algorithm, were done using the implemented Bio Rad CFX manager3.1.software.

2.7. Biochemical analysis

2.7.1.. Liver homogenization and protein quantification

All steps were carried out at 4 °C. Liver samples were homogenized in ice-cold phosphate buffer (100 mmol/L; pH 7.5) using an Ultra-turrax homogenizer. The homogenates were centrifuged at 10.000×g at 4°C for 20 min and the supernatants were then used the estimation of TBARS, PSH, and antioxidant enzymes activity. The total protein level was determined at 595 nm, using bovine serum albumin (BSA) as a standard (Bradford 1976).

2.7.2. Thiobarbituric reactive substances level determination (TBARS)

Liver TBARS, markers of lipid peroxidation were measured by the colorimetric assay of Buege and Aust (1978) using 1,1,3,3-treaethyloxypropane as a standard. The mixture containing liver extract, 15% TCA, 0.375% TBA, and 1 N HCl was heated for 20 min at 95°C (water bath) and then centrifuged. The absorbance of the supernatant was measured at 512 nm and the results were expressed as μmoles/mg protein.

2.7.3. Protein sulfhydryl level determination (PSH)

Liver protein sulfhydryl (PSH) level was determined by the subtraction of non-protein sulfhydryl (NPSH) content from total sulfhydryl content (TSH) (Absorbance1- Absorbance2) (Sedlak and Lindsay, 1968). The concentration of TSH was determined using DTNB (0.01 M) and the absorbance was measured at 412 nm (A1). After precipitation of the sulfhydryl proteins (PSH) by10% TCA, the clear supernatant containing NPSH was treated with DTNB (0.01 M), which was reduced to yellow TNB (5-thiobis (2 nitrobenzoic acid)) and the absorbance was measured at 412 nm (A2). PSH content was expressed as μg per mg of protein.

2.7.4. Antioxidant enzyme activity

Liver catalase (CAT) activity was spectrophotometrically determined by monitoring the disappearance of hydrogen peroxide (H2O2) at 240nm (Claiborne, 1985). Briefly, 10 μL of liver supernatant was mixed with 890 μL phosphate buffer (100 mM, pH 7.5),and 100 μL of

500 mM H2O2 nd The absorbance at 240 nm was measured and the CAT activity was then determined and expressed as μmoles H2O2/min/mg protein.

Superoxide dismutase (SOD) activity was determined based on its ability to inhibit the auto-oxidation of pyrogallol (Marklund and Marklund 1974). Briefly, 200 μL of a cold mixture ethanol: chloroform (1:1, v/v) was added to 250 μL of liver supernatant was and centrifuged at 2500×g for 25 min at 4 °C. The absorbance was immediately measured at 420 nm in the presence of pyrogallol. SOD activity was expressed as units per milligram of protein (U/mg of protein). One unit represents the amount of SOD, which inhibits 50% of pyrogallol autoxidation.

For GST activity, the method of Habig et al. (1974) using 1-chloro-2,4- dinitrobenzene as substrate and glutathione (1 and 4 mmol/L final concentration, respectively) in 100 mmol/L sodium phosphate buffer (pH 7.4) was used. The absorbance was measured at 280nm and the GST activity was expressed as micromoles produced per minute per milligram protein. (μmol/min/g of protein).

All antioxidant enzyme activity assays were performed in conditions of linearity with respect to incubation time.

2.7.5. Insulin-like growth factor-1 levels determination (IGF-I)

Insulin-like growth factor-1 levels were measured in liver using a commercially available ELISA fish kit (m/r IGF-I ELISA; CUSABIO Bio., China). Liver samples were homogenized in 0.1 M PBS (pH 7) (1:10, w/v) and centrifuged at 12000×g for 30 min at 4°C.The supernatants were collected and subsequently assayed for IGF-I following the manufacturer's instructions.

306 **2.8. Histological study**

307 Three fish liver from each tank (nine livers per group) were used for the histological analysis.
308 Three sections from each liver were performed for the analysis of the lipid vacuoles. Samples
309 fixed in 10% buffered formalin were dehydrated through graded alcohol, then xylene and finally
310 embedded in paraffin wax. The paraffin blocks were sectioned at 3 µm, stained with
311 hematoxylin and eosin (H & E) and examined using a light microscope at 400× magnification.

312 **2.9. Statistical analysis**

313 All data were expressed as mean ± standard error of the mean (SEM). In histomorphological
314 evaluation, the standard deviation (SD) was used. Normality and homogeneity of variance were
315 checked using the Shapiro–Wilk and the Levene's tests, respectively. One-way analysis of
316 variance (ANOVA) followed by Duncan's post- hoc multiple range test was used to compare
317 group means at a significance level of $p < 0.05$ and student t-test was used for phytochemical
318 analysis in order to compare the lyophilized and dried AV extracts (LAV and DAV) ($p<0.05$).
319 PCA analysis was used to simplify the data sets, and to determine the correlations between
320 molecular and biochemical parameters and to define the main contributors to the total variance.
321 Statistical analyses were performed using SPSS Statistics v.22 (IBM, Armonk, USA) and using
322 XLSTAT 2.5 (Addinsoft New York, NY).

323 **3. Results**

324 **3.1. Chemical composition of the AV powder**

325 The mean values and SD of the yield of methanolic extract, TPC, TFC, CT, and DPPH of the
326 lyophilized and dried AV powder are presented in Table 3. Compared to the lyophilized sample,
327 the dried powder showed the highest extract yield, TPC, TFC and CT contents, and exhibited
328 the strongest DPPH-radical scavenging activity. For this reason, the dried powder was selected

329 to elucidate its phenolic profile and to investigate its possible effect on lipid metabolism related
330 genes and oxidative stress defense of the sea bream (*Sparus aurata*) under standards rearing
331 conditions.

332 Tentative identification of phenolic compounds of AV dried powder was made based on
333 their mass and UV spectra (Table 4). Overall, the methanolic extract enclosed 3 chromones
334 (Aloeresin A, Aloeresin B and Isoalceresin D) and 6 anthraquinones (Aloin A, Aloin B, 7-
335 hydroxyaloin A, 7-hydroxyaloin B, 8-O-methyl-7-hydroxyaloin A, 8-O-methyl-7-hydroxyaloin
336 B).

337 **3. 2. Effect of AV powder on growth performance**

338 As shown in table 5, the diets supplemented with increasing concentrations of AV powder had
339 no significant effects on the growth parameters. Slight but no significant decreases in WGR and
340 SGR were observed in fish fed with AV 2.5 and 5% supplemented diets. Feed intake did not
341 significantly differ between all groups. However, a slight decrease in feed efficiency was
342 observed in fish fed with 2.5 and 5% AV supplemented diets. The HSI remained unchanged
343 between control and treated groups.

344 **3.3. Effect of AV powder on oxidative stress parameters**

345 The AV powder addition in fish diets at levels of 0.5, 2.5 and 5% dose dependently enhanced
346 ($p<0.05$) the TBARS levels in treated groups compared to control one (Figure. 1A).

347 Concerning antioxidant enzymes, the results indicated that the AV 2.5% powder
348 supplemented diet significantly diminished the SOD activity by 66.20% when compared to the
349 control (Figure. 1B). The level of hepatic PSH was decreased significantly in fish fed with AV
350 2.5% supplemented diet when compared to the control and to the other fish groups fed with AV
351 0.5% and AV 5 % supplemented diets (Figure. 1C).

The hepatic CAT activity was enhanced in fish groups fed with AV 2.5% and AV 5% supplemented diets. The increase was about 2.41 and 1.81 times respectively (Figure. 1D). Regarding the GST, its activity was significantly increased in the fish fed AV 5% supplemented diet (Figure. 1E).

3.4. Effect of AV powder on the IGF-I levels and on the hepatic IGF-I and IGF-II genes expression

The effects of AV supplemented diets on hepatic IGF-I levels and on the gene expression of IGF I and IGF-II involved in the fish growth performance are presented **in Figure. 2.** The IGF-I levels were increased significantly in fish fed with AV 2.5% supplemented diet (Figure. 2A). The molecular analysis of mRNA expression of igf-I gene showed a significant enhancement in fish fed with 0.5%, and 2.5% supplemented diets about 2.34 fold and 2.25 fold respectively (Figure. 2B). Whereas, a 2.6 fold increase of igf-II gene expression was observed in the group treated with the highest concentration of dietary AV 5% as compared to the relative control (Figure. 2B).

3.5. Effect of AV powder on hepatic lipid metabolism-related genes expression

Figure 3 shows the relative changes of hepatic mRNA expression in lipid metabolism-related genes after 8 weeks of feeding with dietary increasing levels of AV powder. The lipogenic gene *fas* expression remained unaltered by dietary AV 0.5% and AV 2.5%; however, it was enhanced in fish fed with diet supplemented with the highest concentration of AV 5% by 5.5 times when compared to the relative control (Figure. 3A). The expression of lipolytic genes (*hsl*) and (*atgl*) and *g6pdh* were significantly reduced in fish fed AV 2.5% supplemented diet (Figure 2A, B). Regarding the (*lpl*) mRNA level, it was significantly downregulated in the fish fed with AV 5% supplemented diet by 47.36% when compared to the relative control. The expression of β-

oxidation related genes was affected in fish fed with A.V 2.5% and 5% but a significant decrease was observed only in the fish fed with AV 2.5% supplemented diet compared to all groups (Figure. 3C). Moreover, *ppar-α* and *hadh* genes expression, was significantly decreased by 53.86% and 58.20%, respectively in fish fed with AV 2.5% when compared to the relative controls. Among fatty acid transporters, *cd36*, which is a class B scavenger receptor that binds many ligands, including fatty acids, and phospholipids, showed a significant downregulation in fish fed with the AV 2.5% supplemented diet. The fatty acid-binding proteins (*fabp*) are a group of fatty acid-trafficking molecules that affect cellular functions. Compared to the control group (Figure 3), the *fabp11* gene expression was reduced by 69.1, 66.8 and 51.3% in fish fed with diets supplemented with 0.5, 2.5 and 5% AV, respectively. The *lxrα*, which is implicated in cholesterol and fatty acid homeostasis was downregulated by 61,6, 58.5 % and 43.6 % in groups treated with AV 0.5, 2.5 and 5%, respectively (Figure 3E).

3.6. Effect of AV on the expression of hepatic genes linked to cellular signaling pathways

In order to investigate whether dietary AV powder influences the expression of genes involved in cell signaling pathways, we analyzed the serine-threonine protein kinase (*akt1*), target of rapamycin (*mtor*) and extracellular signal-regulated protein kinase (erk1/2) mRNA levels. The results showed that the akt11 gene expression was dose dependently decreased by 38.18, 60.51 and 74.10% in fish fed with 0.5, 2.5 and 5% A.V supplemented diets respectively when compared to the relative control group (Figure. 4).

Furthermore, *mtor* mRNA levels were reduced in treated fish groups, although the decrease was only significant in the fish fed with AV 0.5% and AV 2.5% supplemented diets. Regarding *erk1/2* gene, its expression showed a significant decrease in all fish groups fed with experimental diets compared to the control group (Figure. 4). The reduction was evaluated

about 32.07, 68.92 and 64.23% in fish fed with AV 0.5%, AV 2.5% and AV 5% supplemented diets respectively when compared to the relative control group.

3.7. Histological evaluation

Examination of the liver tissue of the control fish showed the characteristic morphology, which consists of hepatocytes (H), sinusoid veins (SV) and an exocrine pancreas or hepatopancreas (HP). The hepatic parenchyma was made up of hepatocytes (H) which were polyhedral cells with a large centrally located nucleus with a few lipid vacuoles (V) (Figure. 5A).

The quantitative histological analysis showed that, in the fish fed with diets supplemented with increasing doses of AV powder for 60 days, there was a significant increase in the number and diameter of the lipid vacuoles compared to the control group (Table 6). Indeed, the number of vacuoles was increased by 1.83, 3.25 and 2.65 times in fish fed with diet supplemented with AV 0.5 %, AV 2.5% and AV 5%, respectively. The group of fish fed with the diet supplemented with AV 2.5% had the highest number of lipid vacuoles. Moreover, the diameter of lipid vacuoles increased by 1.11, 1.64 and 1.71 times in fish fed AV 0.5%, 2.5% and 5%, respectively (Table 6).

3.8. PCA of the biochemical and molecular responses of fish to the AV supplemented diets

PCA is a very useful tool to investigate the effect of the dietary increasing concentrations of A.V. on the molecular and biochemical parameters responses of treated fishes.

PCA is carried out on biochemical (GST, MDA, PSH, IGF-I, CAT and SOD) and molecular parameters (*lpl, atgl, hsl, fas, g6pdh, cd36, fabp11, ppar, hadh, lxr, akt1, erk1 / 2, mtor, igf-i, igf-ii*,) of control and treated fish fed with diets supplemented with AV 0.5%, AV2.5% and AV 5%.

The total variation of the two principal components (CP1 and CP2) is 80.10%. The first PC represented 55.42% of the total variance and was positively correlated with the PSH, *hsl, pparα, hadh, g6pdh, fabp11, lxrα* and *mtor* expression. The PC1 axis clearly separated the untreated control group from the group fed with diet supplemented with AV 2.5% AV.

The PC2 which accounted for 24.68% of the total variance, was positively correlated with the GST, MDA, *fas and igf-ii* (Table 7), and allowed the separation of control group from that fed with diet supplemented with AV 5% (Table 8).

4. Discussion

The use of plant supplements to improve growth, physiological performance and mitigate lipid deposition in fish has recently attracted much attention as an inexpensive, sustainable and effective alternative in aquaculture (Van Hai, 2015; Reverter et al., 2021). However, due to the inherent chemical composition, some plants can exhibit distinct levels of bioactivity and sometimes toxicity; therefore, their detailed phytochemical analysis and the search for optimal concentrations are important to assess their potential for the aquaculture sector (Reverter et al., 2021, Hoseini et al., 2021). Among these plants, AV was widely used as a functional and therapeutic food for both humans and mammals due to its anti-obesity, hypo-glycemic, immune-modulatory and antioxidant properties (Kumar et al., 2019; Maan et al., 2018; Gabriel et al., 2015). Nevertheless, several adverse effects have been explored, which might be relevant to the tested parts of the plant, the time of harvest, the climatic conditions, the extraction or purification processes, the indicated concentration and/or the tested animal system (Tong et al., 2021).

The HPLC-DAD-ESI-MS profiling of the methanol extract of the dried AV, allowed the identification of 9 compounds including 3 chromones (Aloeresin A, Aloeresin B and

Isoalceresin D) and 6 anthraquinones (Aloin A, Aloin B, 7-hydroxyaloin A, 7-hydroxyaloin B, 8-O-methyl-7-hydroxyaloin A, 8-O-methyl-7-hydroxyaloin B). These compounds have already been reported as distinctive phenolic compounds in different Aloe species (Añibarro-Ortega et al., 2019; Li et al., 2018) and most of them are credited with a long list of biological activities including antioxidant, anti-bacterial, antimycoplasmic and anti-cancer activities, among others (López et al., 2013).

In the current study, AV supplementation had no beneficial effects on fish growth (WG, WGR, and SGR) and feed utilization (FI, FCR, FER) indexes. Growth-promoting effects of herbal supplementation depends on the fish species, the type, and the concentration of the used material (Hoseini et al., 2021). For example, Mehrabi et al. (2019) showed that dietary supplementation of AV in the diet of rainbow trout at 5, 10 and 15% for 8 weeks significantly improved their growth performance. However, Gabriel et al. (2015) demonstrated that the inclusion of the plant at 0.5% and 2% improved growth indexes in GIFT-strain tilapia fish, while the higher dietary AV concentration (4%) did not affect these parameters. Growth is controlled being the most influential genes those of insulin-like growth factors (IGF-II and I) as they are the core of the hypothalamic–pituitary–somatotropic axis (HPS) together with the growth hormone (GH) (Triantaphyllopoulos et al., 2020). The effects of dietary conditions on insulin growth factors gene expression was reported to be a great potential to optimize fish growth rate but in the present study, the increase in IGF-I and IGF-II gene's expression and levels by AV diets was not associated with the enhancement of gilthead sea bream growth indexes. A possibility to explain these differences, it could be that although hepatic IGFs are enhanced by AV, the experimental period may not sufficient to show a significant induction of somatic growth by these peptides and the growth rates of fish of the size used in this experiment

466 are slower than that of small juveniles (Myrick and Cech 2000). Furthermore, the endocrine
467 mechanisms that mediate changes in the GH/IGF growth axis under different metabolic or
468 nutritional states are not fully understood at present because the growth signaling system is a
469 very complicated network and many factors are involved in this phenomenon process
470 (Triantaphyllopoulos et al., 2020).

471 The liver is the central organ for lipid metabolism and detoxification (Meng et al., 2019). Here,
472 the observations of histological structure of the liver in the research support of the above
473 conclusion. There was an abnormal lipid storage and deposit accompanied by an alteration in
474 lipid metabolism at a transcriptional level, and oxidative stress in fish fed with diets
475 supplemented with the higher concentrations of the used AV powder. The increase in the
476 number and diameter of hepatic lipid vacuoles in fish fed with the AV 2.5% and AV 5%
477 powder supplemented diets was observed. The overload of lipids, mainly triacylglycerol (TG),
478 in the cytoplasm of hepatocytes (> 60%) resulted in severe steatosis. This might be due to the
479 presence of transformed constituents in AV (such as aloin) that may block hepatic metabolic
480 enzymes (Rabe et al., 2005). Similar results have been observed in gilthead sea bream fish fed
481 with 60% soybean oilas replacement (Bouraoui et al., 2011). Several cases of AV-induced toxic
482 hepatitis in human patients have been reported in recent years. However, the involved
483 mechanisms have not yet been described in the literature (Lee et al., 2014). Several studies have
484 shown that AV is considered as anti-obesogenic, Nevertheless, the effects of some bioactive
485 fitocompounds like phytoestrogens (i.e., genistein) also present in AV can have different effects
486 on lipid metabolism in fish and in mammals depending on the tissue (Balbuena-Pecino et al.,
487 2020). Even contradictory results are found in different mammalian models (Park et al., 2009;
488 Grossini et al., 2018).

The disruption in lipid metabolism promotes the progression of liver damage. Regarding the "multiple hit process" during liver injuries, the first "hit" is the “accumulation of fat”, suggesting that the change in lipids occurs as a first step in hepatic damage (Yang et al., 2019). Lipid accumulation results from the balance between lipogenesis and lipolysis, and many genes are involved in this process (Meng et al., 2019). In this sense, *fas* is known to play crucial roles in lipogenesis by catalyzing the synthesis of saturated long chain fatty acids from acetyl-CoA, malonyl-CoA, and NADPH thus, can have adverse influence on fish liver function because it can induce lipid accumulation (Bou et al., 2014). In our study, (*fas*) expression was up-regulated in fish fed with the AV 5% supplemented diet, although not accompanied by *6gpdh* expression increase, the NADPH supplying enzyme. In addition, *lpl* gene expression was down-regulated in fish fed with AV 5%. These results suggest that dietary AV powder supplementation could enhance hepatic *de novo* lipogenesis while reducing lipolysis of plasma TG. These observations were in line with those of Rahoui et al (2018) showing that impaired lipolysis and a reduced *hsl* expression would promote the storage of excess lipids in rats. In other study, it has been reported that AV gel treatment inhibits the porcine pancreatic lipase activity *in-vitro* through its binding ability to both the free-enzyme and the enzyme-substrate complex (Taukoorah and Mahomoodally, 2016). In fish, several transcription factors play an intermediary role in lipid homeostasis, by controlling the gene transcription of the enzymes involved in lipogenesis and lipolytic pathways (Cruz-Garcia et al., 2011). Considering the transcriptional factors studied here, it has been described that *lxrα* is involved in TG breakdown in fish tissues (Cruz-Garcia et al., 2012), and its induction increases lipid efflux and decreases lipoprotein uptake, thus avoiding excessive lipid accumulation (Kidani et al., 2012). *pparα* stimulates the mobilization and the degradation of FAs by β-oxidation in tissues particularly in liver (Wei et al., 2017). In

the current study, *lxrα* gene was significantly down-regulated by the three-tested concentration of AV supplemented diet and *pparα* gene was significantly down-regulated by AV 2.5% powder supplemented diet. In parallel a down-regulation of gene expression of *hdadh* was showed, an enzyme involved in FA β-oxidation (Paredes et al., 2015) together with a decrease in *fabp11a* expression, a binding protein known to facilitate the entry of FAs into the cell for oxidation or storage. The mentioned results are in agreement with the work of Tseng-Crank et al (2013), who reported that UP780 (the combination of AV inner gel powder standardized containing 2%-4% aloesin) reduced FA β-oxidation by the inhibition of the *cpta1a*, *pparα* transcription factor and some FA transporters genes' expression. The second hit during liver injury was considered to be "oxidative stress" (Ramachandran and Jaeschke 2018). In healthy hepatic cells, an appropriate enzymatic and non-enzymatic antioxidant system has been described as a mechanism to eliminate excessive ROS. Among the most frequently, used biomarkers of oxidative stress are the enzymes SOD, CAT and GST. The inhibition of SOD activity may be due to the direct interaction between the enzyme and the aloin from AV extracts. Aloin is known to inhibit the action of the metalloprotease by interacting with zinc and/or calcium at the secondary site of the enzyme, which leads to a destabilization of its structure, which in turn enhances the production of O_2. radicals inside the cell and as a result of cell injury (Liang et al., 2021). Several studies have shown the prooxidant effect of this molecule from AV. It reduces (Fe^{3+}) to (Fe^{2+}) from Fenton reaction, which increases the generation of the hydroxyl radical (OH.) and (H_2O_2) (Nowak et al., 2021). The enhancement of CAT activity by AV treatment could be due to the presence of free radicals like H_2O_2, generated by the effects of aloin and its derivatives. PSHs plays a protective role against free radicals because of their sulfhydryl SH unit. In this study, the level of PSH proteins was reduced by the treatment of AV

535 at 2.5% level of inclusion. This could be due to the presence of excess of H_2O_2. Sutariya and
536 Patel (2017) demonstrated that excessive H_2O_2 could block SH groups leading to the loss of SH
537 units' reactivity, which ultimately induce toxicity (Smith et al., 2019). GSTs catalyze the
538 conjugation of reduced glutathione (GSH) via the SH group, to electrophilic centers on
539 endogenous compounds (Pavlidi et al., 2018). The GST activity induction in fish fed with AV
540 5% supplemented diet could play an important role in the protection against harmful aloin
541 metabolites generated after the metabolization (Yan et al., 2016). Several studies have shown
542 that the depletion of antioxidant enzymes leads to ROS accumulation and the development of
543 liver disease (Kim et al., 2020). The reduction of SOD activity and the increase of TBARS
544 levels in fish fed with diet supplemented higher concentrations of AV powder could be the
545 consequence of the high generation of ROS like the hydroxyl radical generated by aloin. Being
546 the specific target of ROS, macromolecules namely lipids could be easily oxidized to produce
547 malonedialdehyde (MDA), a marker of lipid peroxidation and induce structural and functional
548 alterations of cell bio-membranes (Yan et al., 2015). Support to this assumption is given by
549 (Buenz, 2008) who showed that the treatment of Jurkat cells with aloin, dose-dependently
550 reduced cell size, disrupt and blocked the cell cycle at the G2/M phase. Nevertheless, after 0.5%
551 AV treatment the parameters related to hepatic oxidative status were not significantly affected.

552 Concerning signaling pathways, *akt* and *mtor* are molecules known, among others, to
553 improve the efficiency of global protein synthesis and stimulate hepatic lipogenesis (Zhang et
554 al., 2019). *mtorc1* is a serine/threonine kinase that plays a central role in many processes such
555 as protein and lipid synthesis and, at the same time, it also suppresses autophagy, therefore
556 controlling the balance between cell anabolic and catabolic pathways (Saxton et al., 2017).
557 Here, the incorporation of AV powder in the fish diet induced significantly a down-regulation

of *mtor*. It has been shown that mtorc1 promotes *de novo* lipid synthesis through the activation of sterol responsive element binding protein (*srebp1c*), a transcription factor that activates numerous genes involved in lipid biosynthesis (Pan et al., 2019). On the other hand, the reduction of *mtorc1* gene expression causes an impairment in oxidative capacity, and induces autophagy in tilapia (Han et al., 2020). Furthermore, the *mtor* pathway played an important role in the inflammatory response in fish (Tan et al., 2018). The inhibition of *mtor* signaling pathway increased proinflammatory cytokines such as *il-6, tnf-α,* inducing apoptosis in hybrid grouper (Tan et al., 2018). Therefore, the lower levels of *mtor* observed in our study could contribute to the impairment of oxidative status and hepatic injury in gilthead sea bream. However, the decrease in *mtor* could be in fact a tissue response to reduce fat accumulation, since several studies showed that rapamycin, the inhibitor of *mtorc1,* inhibits lipogenenic enzymes in trout (Dai et al., 2013), and also alleviates hepatic steatosis caused by fructose in zebrafish (Han et al., 2020). The protein kinase B (*akt*) activation has been shown to increase lipogenic genes expression. In fact, the inhibition of *akt* activation blocks the increase in mRNA expression of the *srebp1c* gene (Smith et al., 2008). Our findings indicate that hepatic *akt* gene expression was decreased by AV treatment reflecting presumably an adaptive cellular mechanism to also reduce liver steatosis. A recent transcriptional study showed that the suppression of *akt* driven Ribosomal protein (RPS) phosphorylation and *srebp1c/ fas* mediated lipogenes is alleviate hepatic steatosis (Zhang et al., 2019). Next, *erk1/2* are part of the family of Mitogen activated protein kinases (MAP kinases), proteins that play an important role in cell division, growth and proliferation (Chen et al., 2018; Mahali et al., 2014) and stimulate the phosphorylation of *srebp1c*. In the current study, an inhibition of the expression of *erk1/2* by the tested concentrations of AV was noted contributing to the regulation of lipogenesis. Alternatively,

such inhibition might be attributed, at least in part, to the active AV component aloin as previously reported in mouse (Zhong et al., 2019).

In summary, gilthead sea bream (Sparus aurata) fed with diet supplemented with higher concentrations of AV powder showed an induction of oxidative stress and lipid peroxidation, alteration of the expression of genes involved in lipid metabolism and cellular signaling pathways, and high hepatic lipid deposition as revealed by histological analysis.

The principal component analysis of the molecular, biochemical and histological responses of the fish (*Sparus aurata*) fed with the increasing concentrations of AV showed that the principal component 1 (PC1) is positively correlated with antioxidant markers (SOD and PSH) and expression levels of genes involved in lipid metabolism and regulation (*hsl, pparα, hadh, g6pdh, lxrα, fabp11, mtor*). This means that by adding the AV 2.5% supplemented diet, the animal responds through several biochemical and molecular pathways to combat this addition. The principal component 2 (PC2 axis) (24.68% of the total variance) is positively correlated with the biochemical variables (GST and MDA) and the lipogenic genes (*fas*) and (*igf-II*) and clearly separate the control group from the AV 5% group. This means that increasing AV concentrations in the diet of fish altered the biochemical and molecular parameters including the disruption of antioxidant status (PSH and SOD), extended lipid peroxidation. These alterations are responsible for an abnormal lipid metabolism in hepatocytes by reducing the expression of genes involved in lipolysis (*hsl, atgl*), those involved in the catabolism of FAs (*ppar, hadh*) in particular AV 2.5% and the increase in that of genes involved in lipogenesis such as *fas* by the AV5% then causing hepatic steatosis.

5. Conclusions

In summary, we have evaluated the effects of different concentrations of dietary AV supplementation on gilthead sea bream growth performance, antioxidant status, liver histology and lipid metabolism related genes' expression. We found that supplementation at 0.5% enhanced hepatic IGFs levels and expression without affecting the other studied parameters. However, AV at 2.5 % and 5% caused oxidative stress, and reduced the efficacy of liver FA catabolism, and cell survival pathways-related genes, while increased FA synthesis-related genes' expression, which is associated with hepatocellular excessive lipid accumulation and as a result, hepatic steatosis, and this might be due to the identified anthraquinones in AV powder.

Author contributions

The authors thank the participants who gave their time to the trial. Jamel JEBALI and Isabel NAVARRO designed, supervised the study and revise manuscript. Afef AMRI designed, performed experiments, analyzed data and co-wrote the paper. Zied BOURAOUI contributed to animal experiments, samples collection and analyzed data. Sara BALBUENA-PECINO and Encarni CAPILA contributed to molecular analyses and revise manuscript. Hamadi GUERBEJ carried out the rearing work. Karim HOSNI supervised the phytochemical analysis and revise manuscript. Tahar GHARRED and Zohra HAOUAS contributed to the histological and statistical analysis of the results. All authors analyzed the results and contributed to the manuscript writing, and approved the final version of the manuscript.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declaration of Interest Statement

625 The authors confirm that there are no conflicts of interest in this work.

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635 **Ethical approval**

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FIGURE CAPTIONS

Figure. 1: Hepatic oxidative stress parameters of gilthead sea bream fishes fed with diets containing 0, 0.5%, 2.5% and 5% of dietary *Aloe vera* (AV) powder for eight weeks. (A) Catalase (CAT) and Glutathione-S-trensferase (GST), (B) Protein sulfhydryl (PSH) and Thiobarbituric acid reactive substance (TBARS) levels Data represent means ± SEM). (10 fish/treatment). Different letters indicate significant difference among between fish groups (one way ANOVA and Duncan's test, p< 0, 05).

Figure. 2: Growth factors (A) hepatic insulin-like growth factor level and (B) gene expression of insulin-like growth factor-I and II of gilthead sea bream fed the control and the experimental diets supplemented with 0.5%, 2.5% and 5% *Aloe vera* powder for 8 weeks. mRNA expression values were normalized to reference genes (*ef1a* and *rps18*) expressed as a ratio of the control groups. Data represent means ± SEM. Different letters indicate significant difference between fish groups (one way ANOVA and Duncan's test, p< 0, 05).

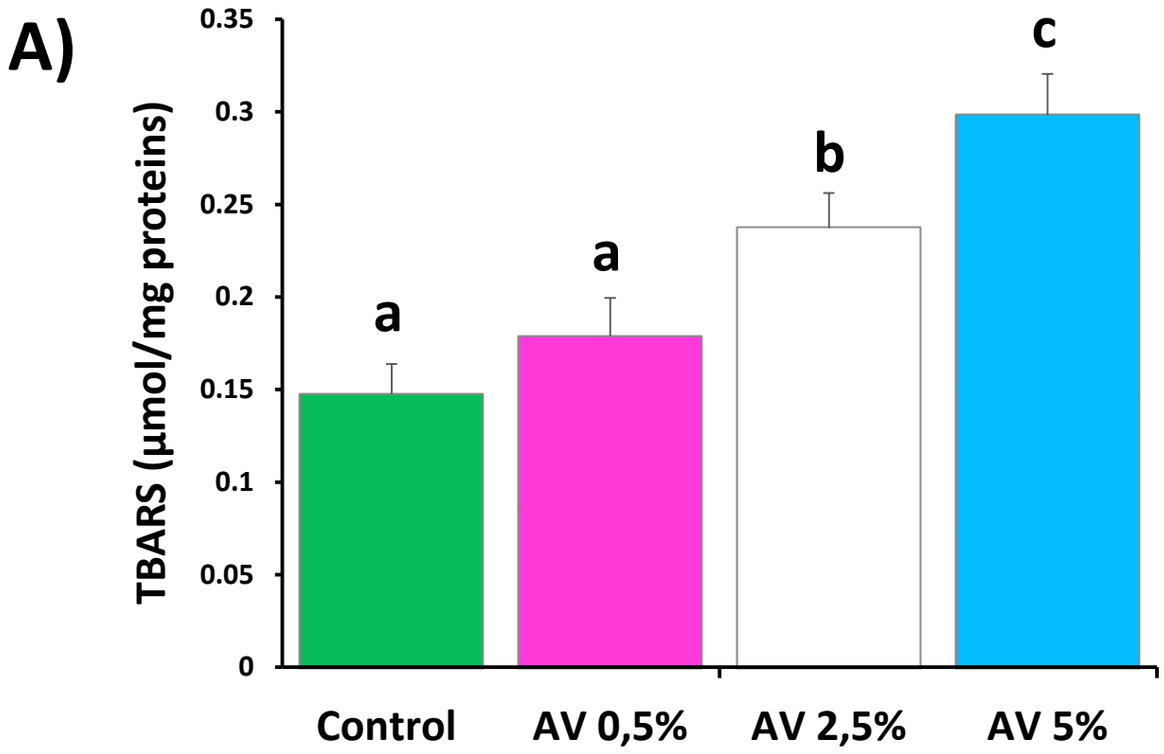
Figure. 3: Hepatic expression of genes involved in lipid metabolism in gilthead sea bream fed the control and the experimental diets supplemented with 0.5%, 2.5% and 5% *Aloe vera* powder for 8 weeks. (A): genes coding for enzymes related to lipogenesis [Fatty acid synthase (*fas*) and glucose-6-phosphate dehydrogenase (*g6pdh*)], (B): genes of enzymes involved in lipolysis [Lipoprotein lipase (*lpl*), hormone-sensitive lipase (*hsl*) and Adipose triglyceride lipase (*atgl*)], (C): gene of transcription factors (Peroxisome proliferator-activated receptor (*pparα*) and selected enzyme (Hydroxyacyl-Coenzyme A dehydrogenase (*hadh*) involved in β-oxidation pathway (D) genes of fatty acids transporters [cluster of differentiation 36 and fatty-acid-binding proteins (*fabp11*)] and (E): gene of transcription factor involved in cholesterol and fatty acid homeostasis [Liver X receptor alpha (*lxrα*)]. mRNA expression values were normalized to

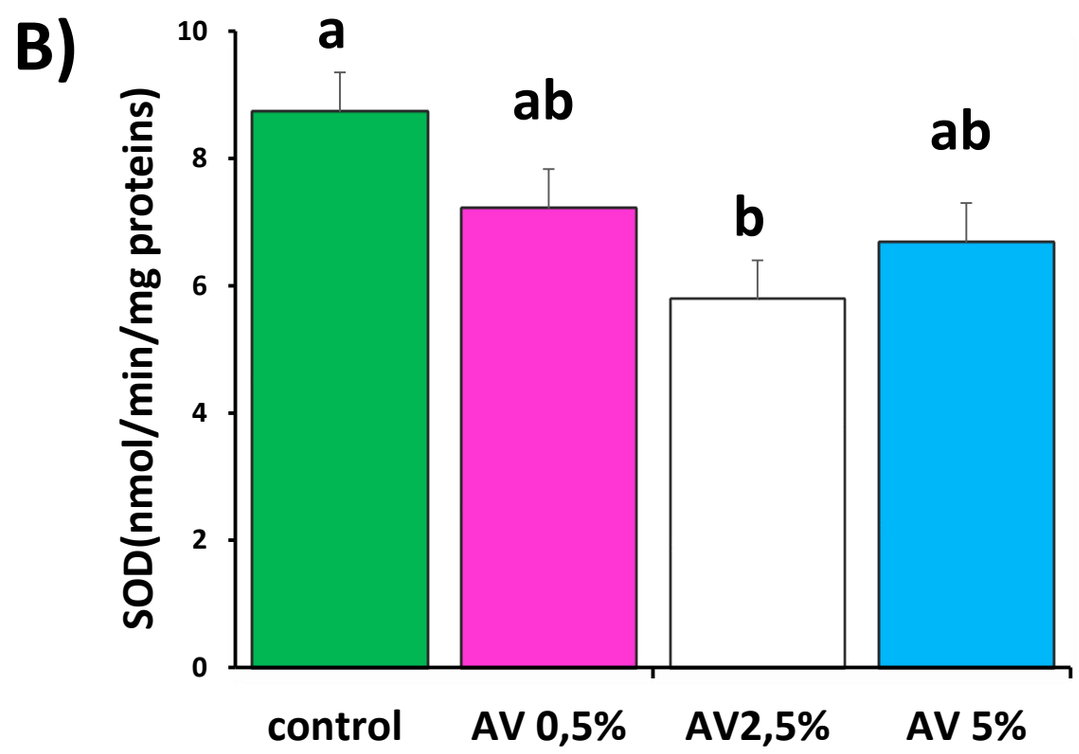
reference genes (*ef1a* and *rps18*) expressed as a ratio of the control groups. Data represent means ± SEM. (6 fish/treatment). Different letters indicate significant difference between fish groups (one way ANOVA and Duncan's test, p< 0, 05).

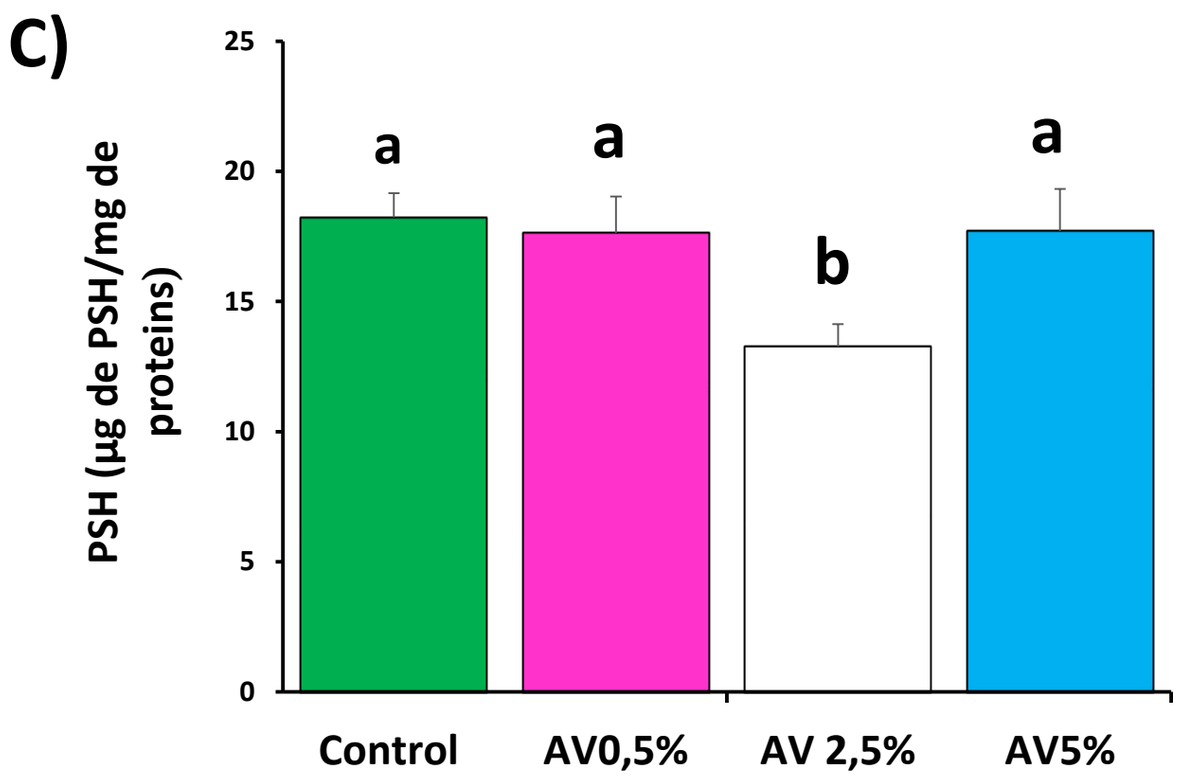
Figure. 4: Gene Expression of protein kinases involved in cellular signaling pathways. [RAC-alpha serine/threonine-protein kinase of gilthead sea bream fed the control and the experimental diets supplemented with 0.5%, 2.5% and 5% *Aloe vera* powder for 8 weeks. (*akt1*), mammalian target of rapamycin (*mtor*) and extracellular-signal-regulated kinase (*erk1/2*). mRNA expression values were normalized to housekeeping genes (*ef1a* and *rps18*) expressed as a ratio of the control groups. Data represent means ± SEM (6 fish/treatment). Different letters indicate significant difference between fish groups (one way ANOVA and Duncan's test, p< 0, 05).

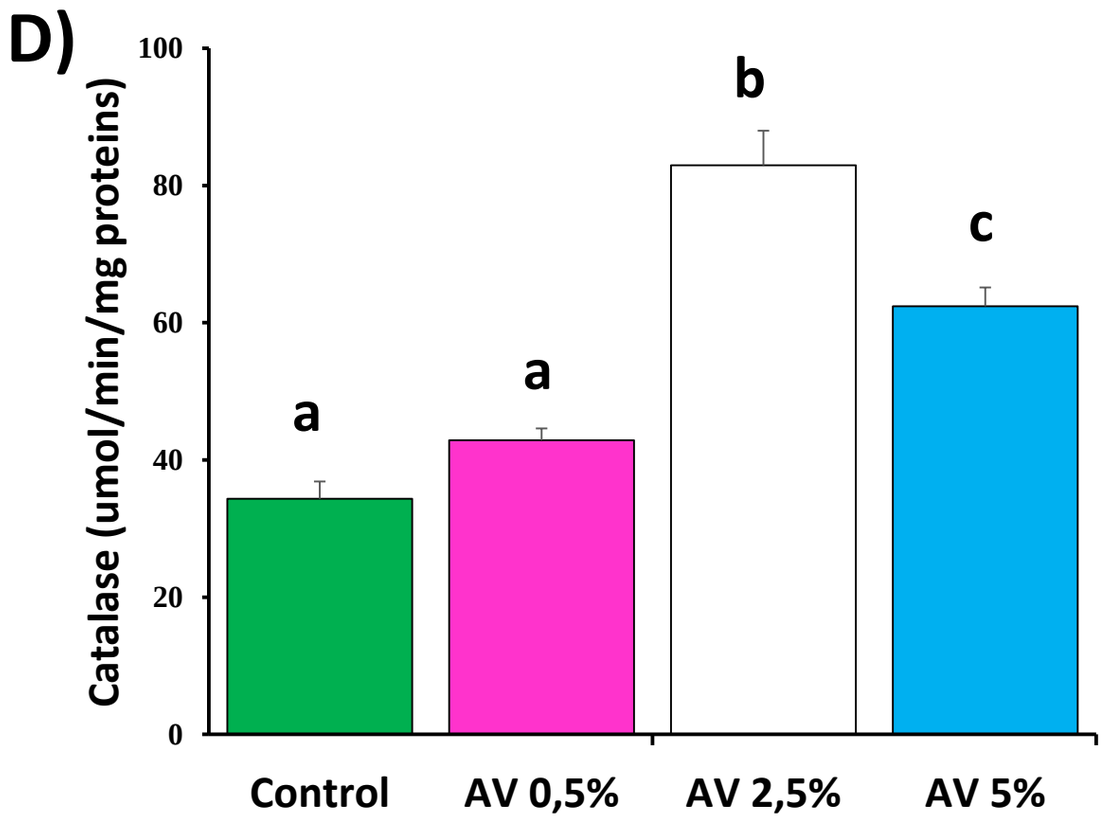
Figure. 5: Hematoxylin-eosin staining of liver tissues of gilthead sea bream fed with control (A) and experimental diets supplemented with (B) a): 0.5% *Aloe vera* powder, (C): 2.5% *Aloe vera* powder (D): 5% *Aloe vera* powder for 60 days. HP hepatopancreas, H hepatocytes, V vacuolization, the magnification was × 40 N = 9 fish/treatment and 3 sections per fish were analyzed.

Figure. 6: Principal component analysis (PCA) performed on the biochemical and molecular variables of gilthead sea bream fed the control and the experimental diets supplemented with 0.5%, 2.5% and 5% *Aloe vera* powder for 8 weeks.









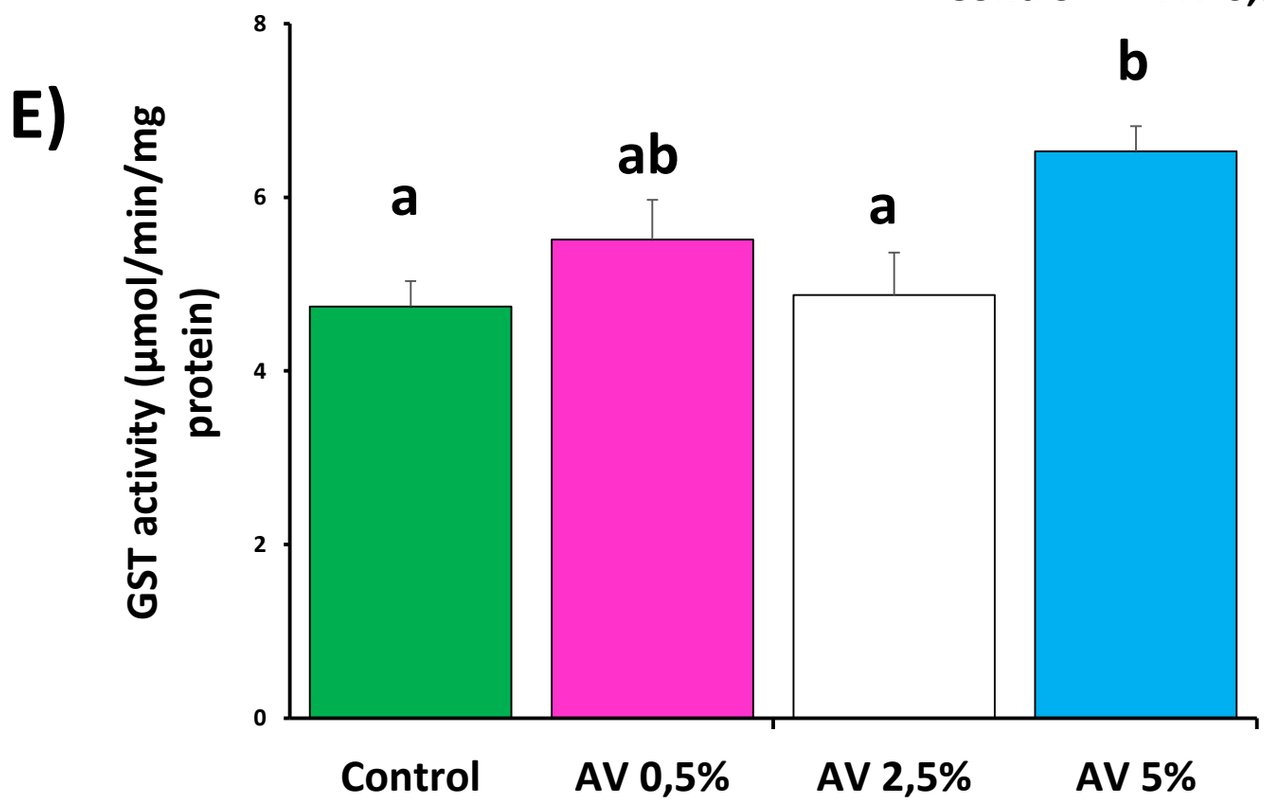


Figure.1

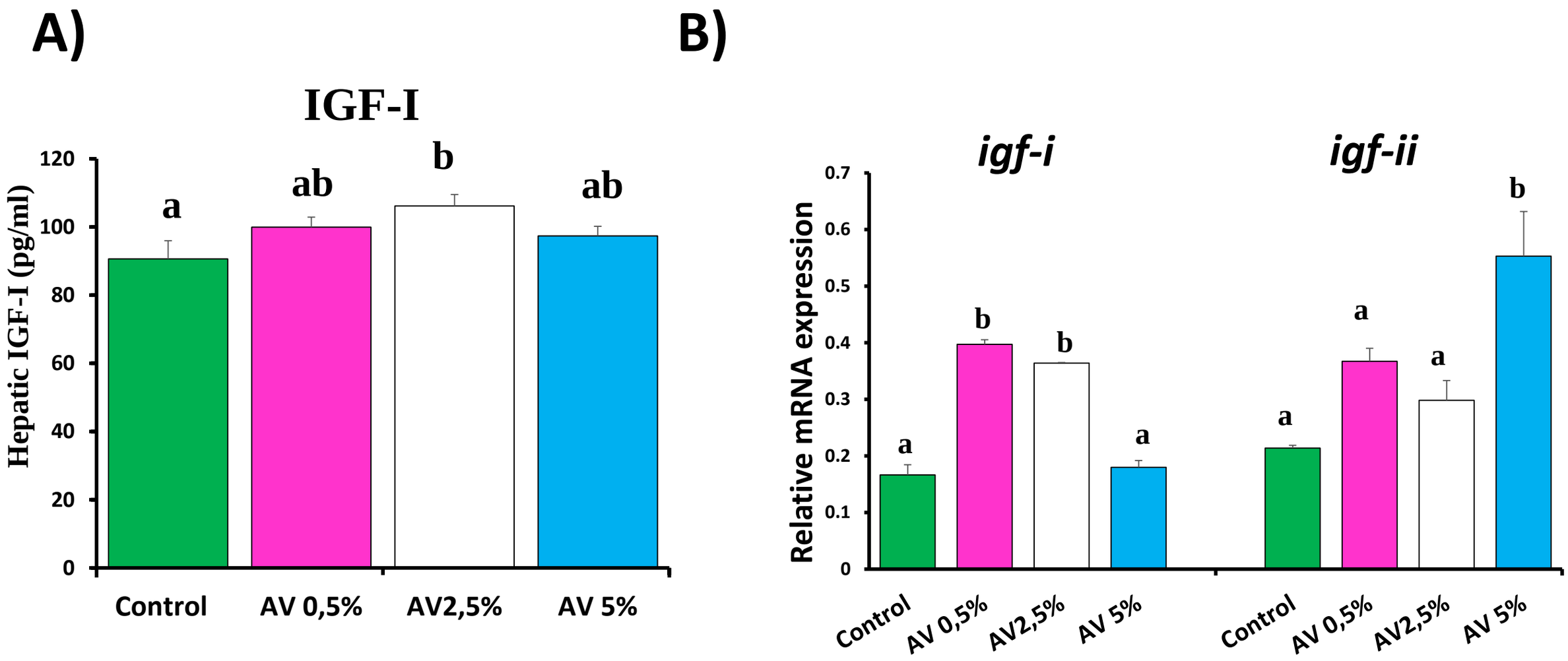
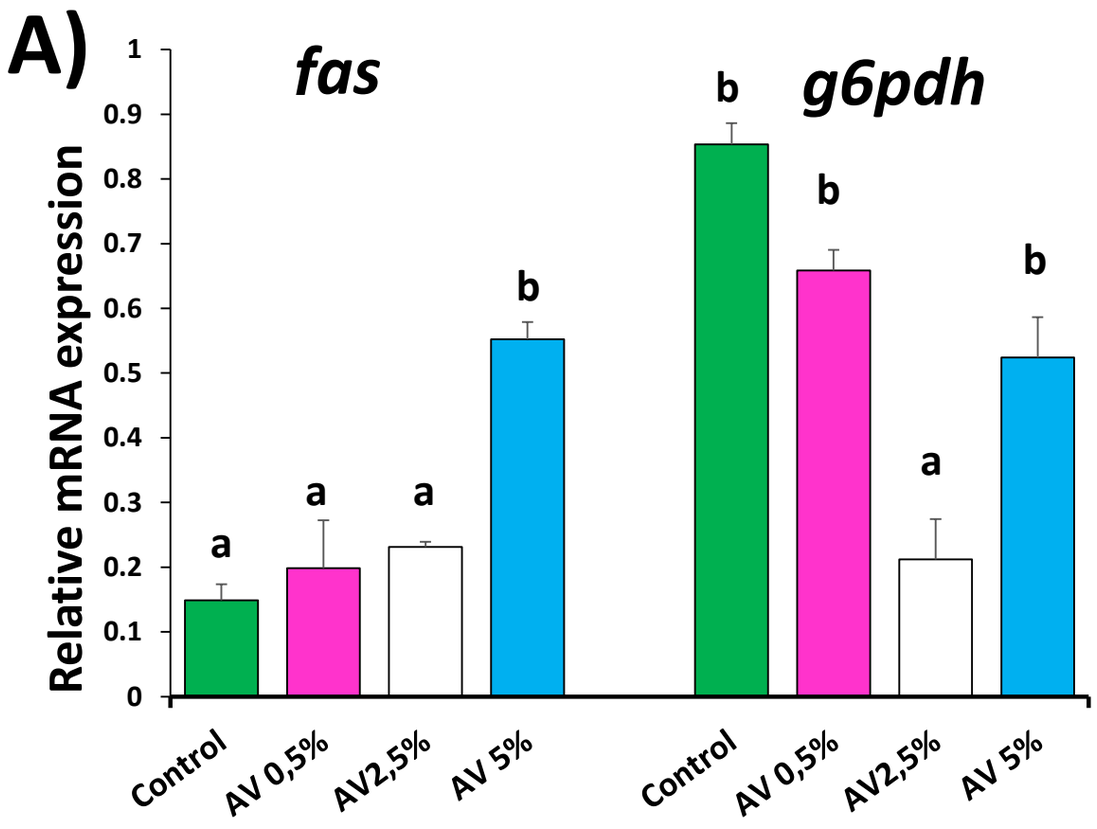
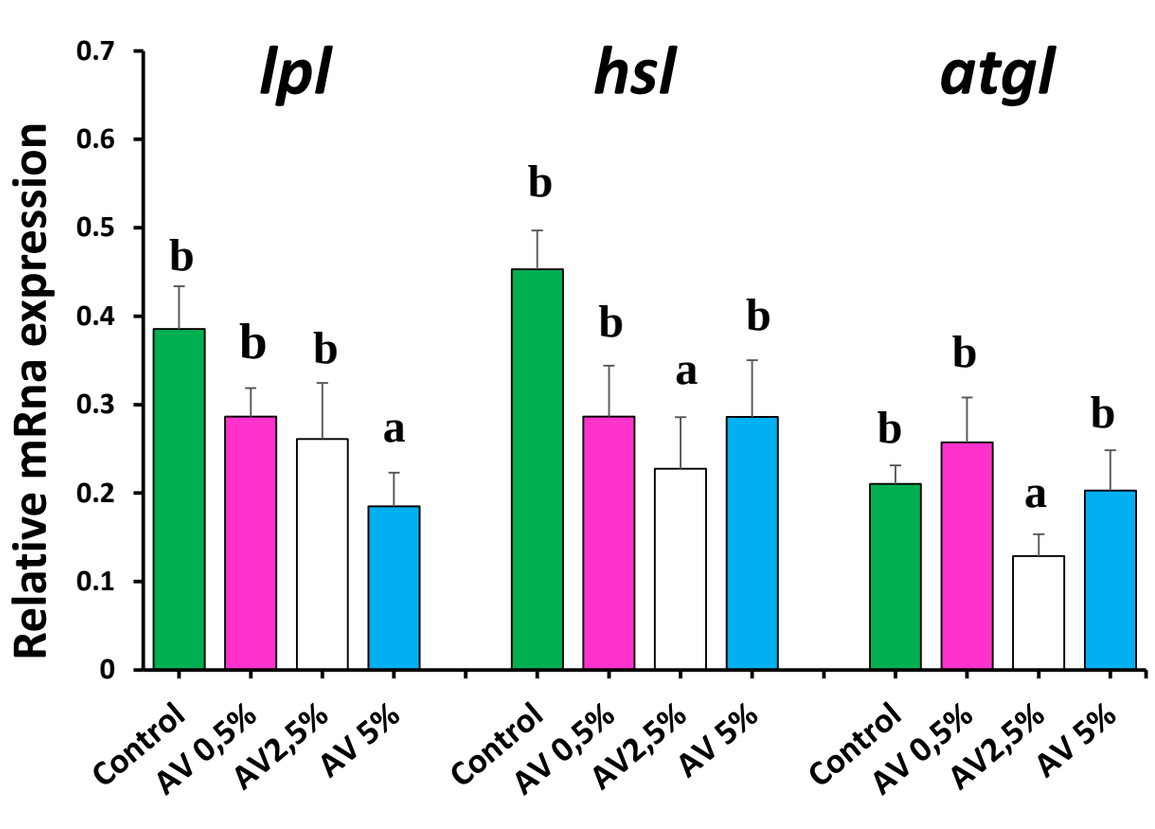
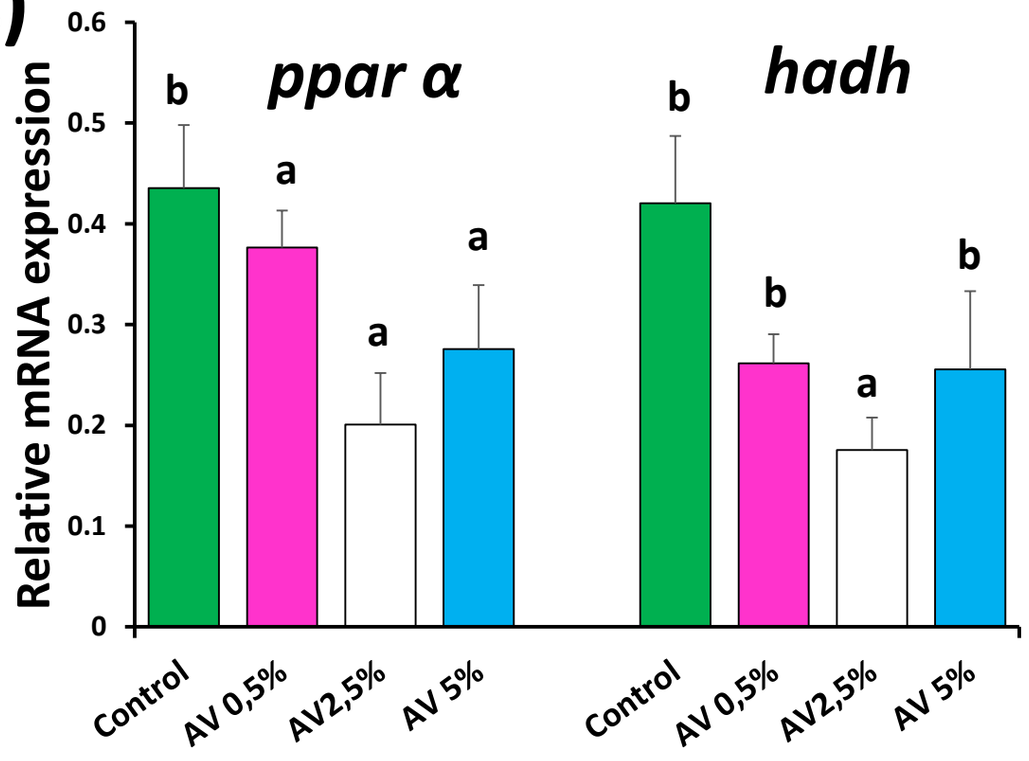
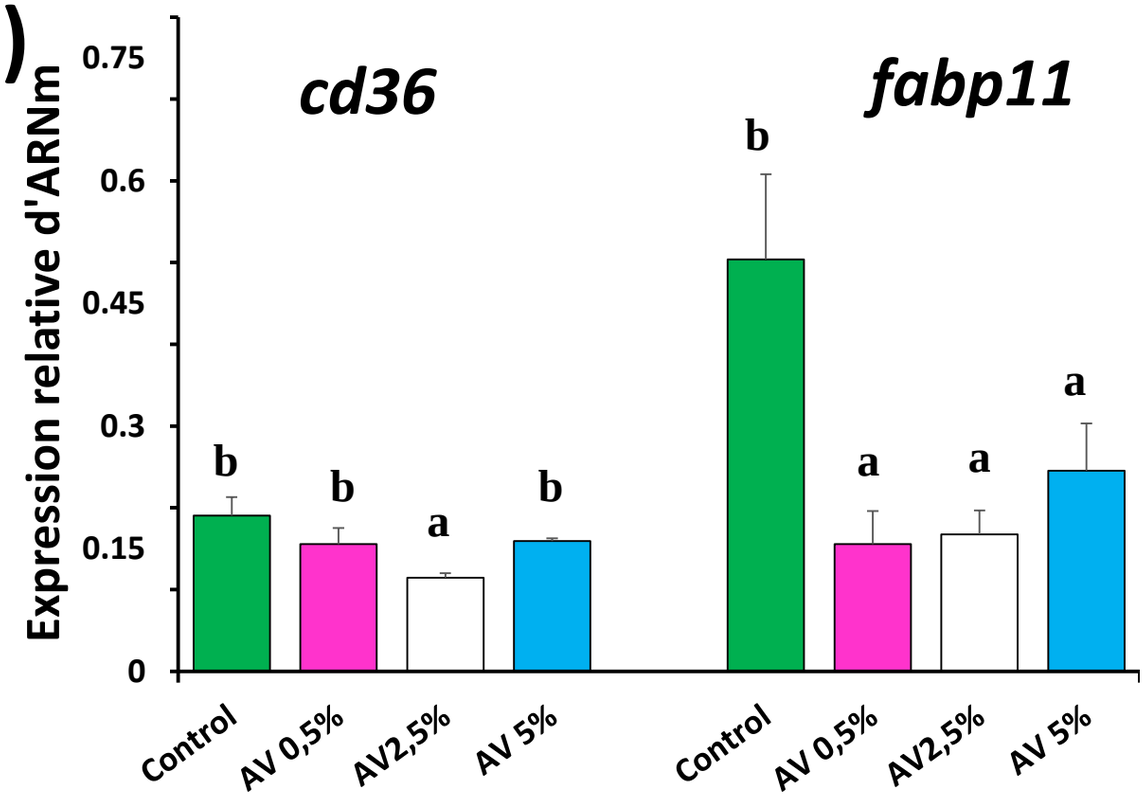

A)
IGF-I
Hepatic IGF-I (pg/ml)
120
100
80
60
40
20
0
a
ab
b
ab
Control
AV 0,5%
AV2,5%
AV 5%
B)
igf-i
igf-ii
Relative mRNA expression
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
a
b
b
a
a
a
a
b
Control
AV 0,5%
AV2,5%
AV 5%
Control
AV 0,5%
AV2,5%
AV 5%

Figure.2


A)
fas
g6pdh
Relative mRNA expression
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
a
a
a
b
b
b
a
b
Control
AV 0,5%
AV2,5%
AV 5%
Control
AV 0,5%
AV2,5%
AV 5%


B)
lpl
hsl
atgl
Relative mRna expression
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
b
b
b
a
b
b
b
a
b
b
a
b
Control
AV 0,5%
AV2,5%
AV 5%
Control
AV 0,5%
AV2,5%
AV 5%
Control
AV 0,5%
AV2,5%
AV 5%


C)
ppar α
hadh
Relative mRNA expression
0
0.1
0.2
0.3
0.4
0.5
0.6
b
a
a
a
b
b
a
b
Control
AV 0,5%
AV2,5%
AV 5%
Control
AV 0,5%
AV2,5%
AV 5%


D)
cd36
fabp11
Expression relative d'ARNm
0
0.15
0.3
0.45
0.6
0.75
b
b
a
b
b
a
a
a
Control
AV 0,5%
AV2,5%
AV 5%
Control
AV 0,5%
AV2,5%
AV 5%

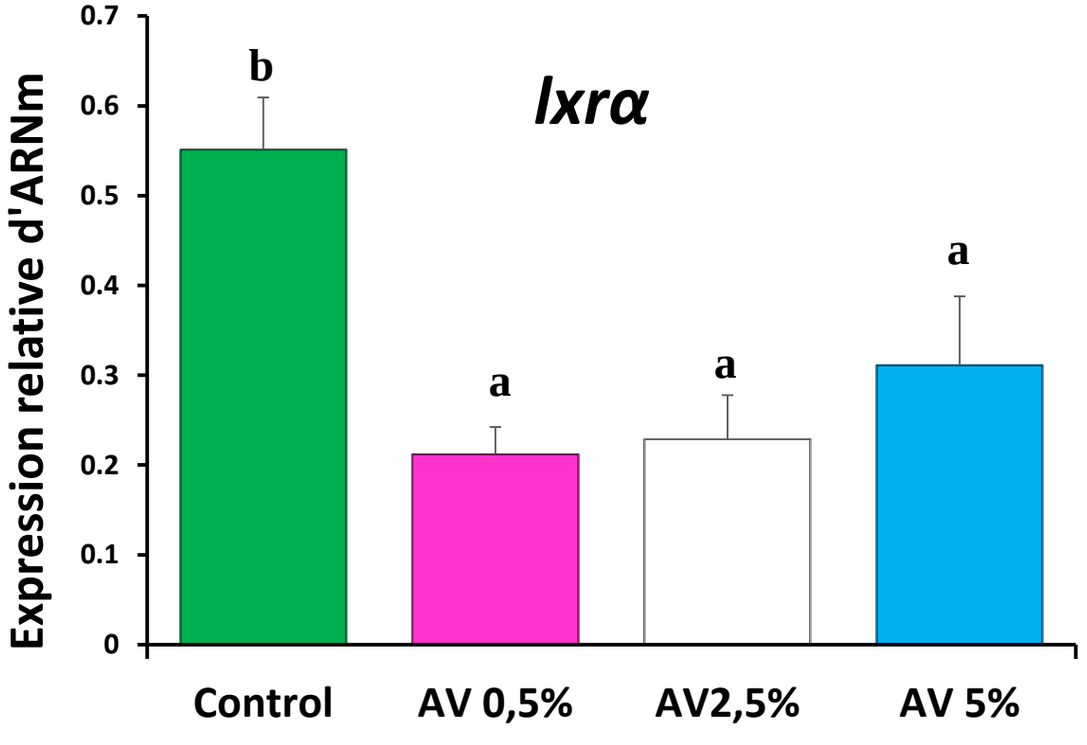

E)
lxrα
Expression relative d'ARNm
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
b
a
a
a
Control
AV 0,5%
AV2,5%
AV 5%

Figure.3

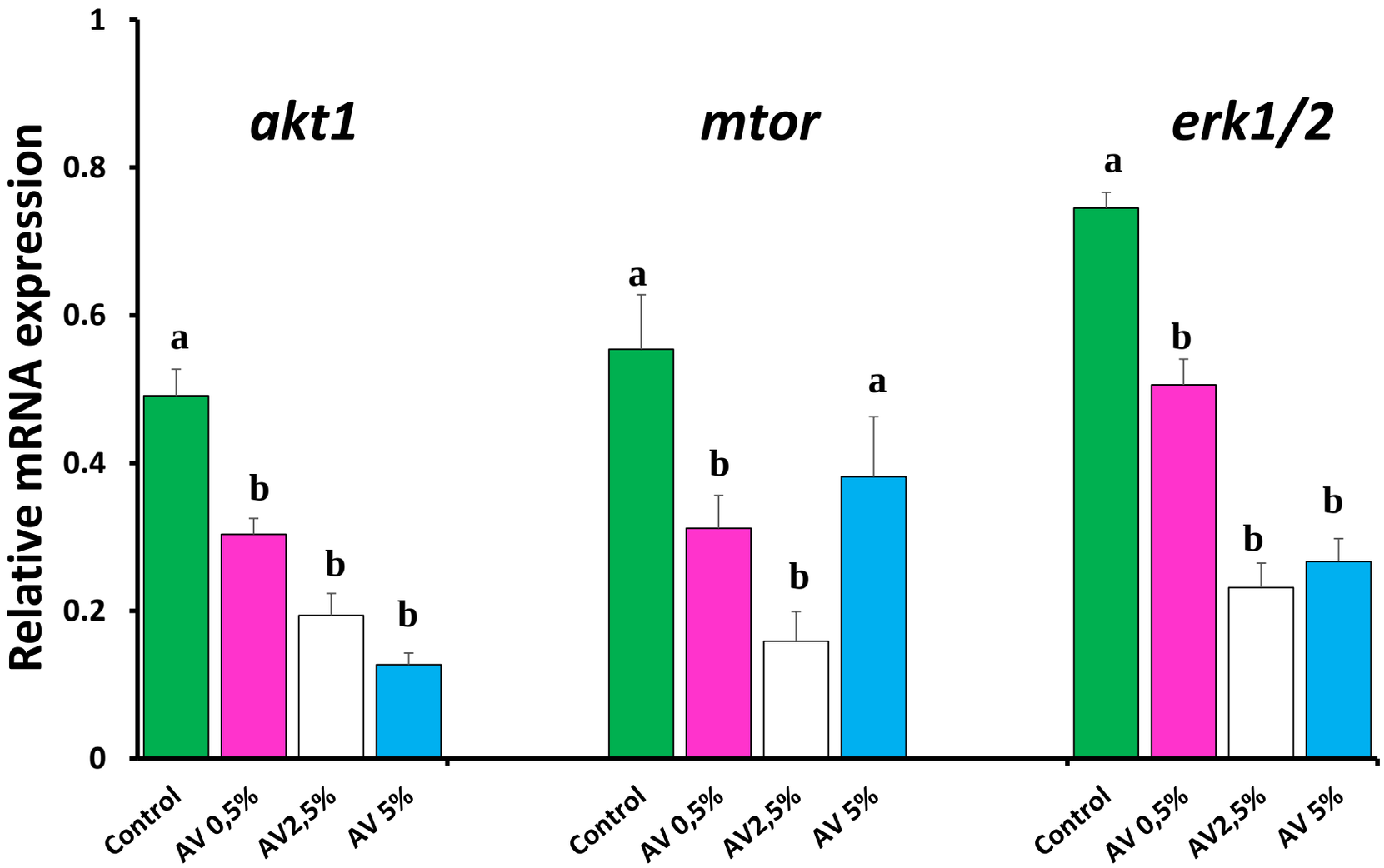
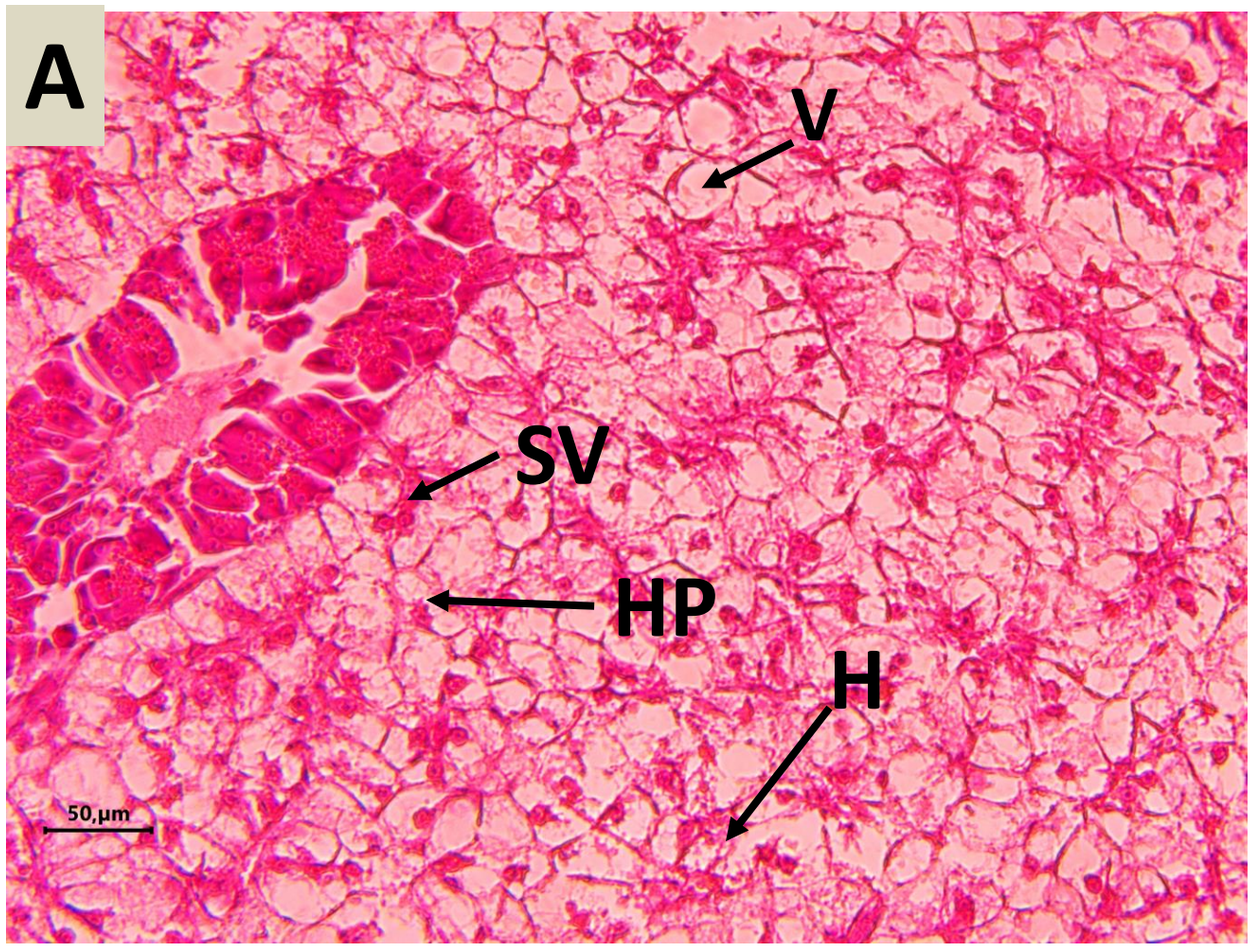
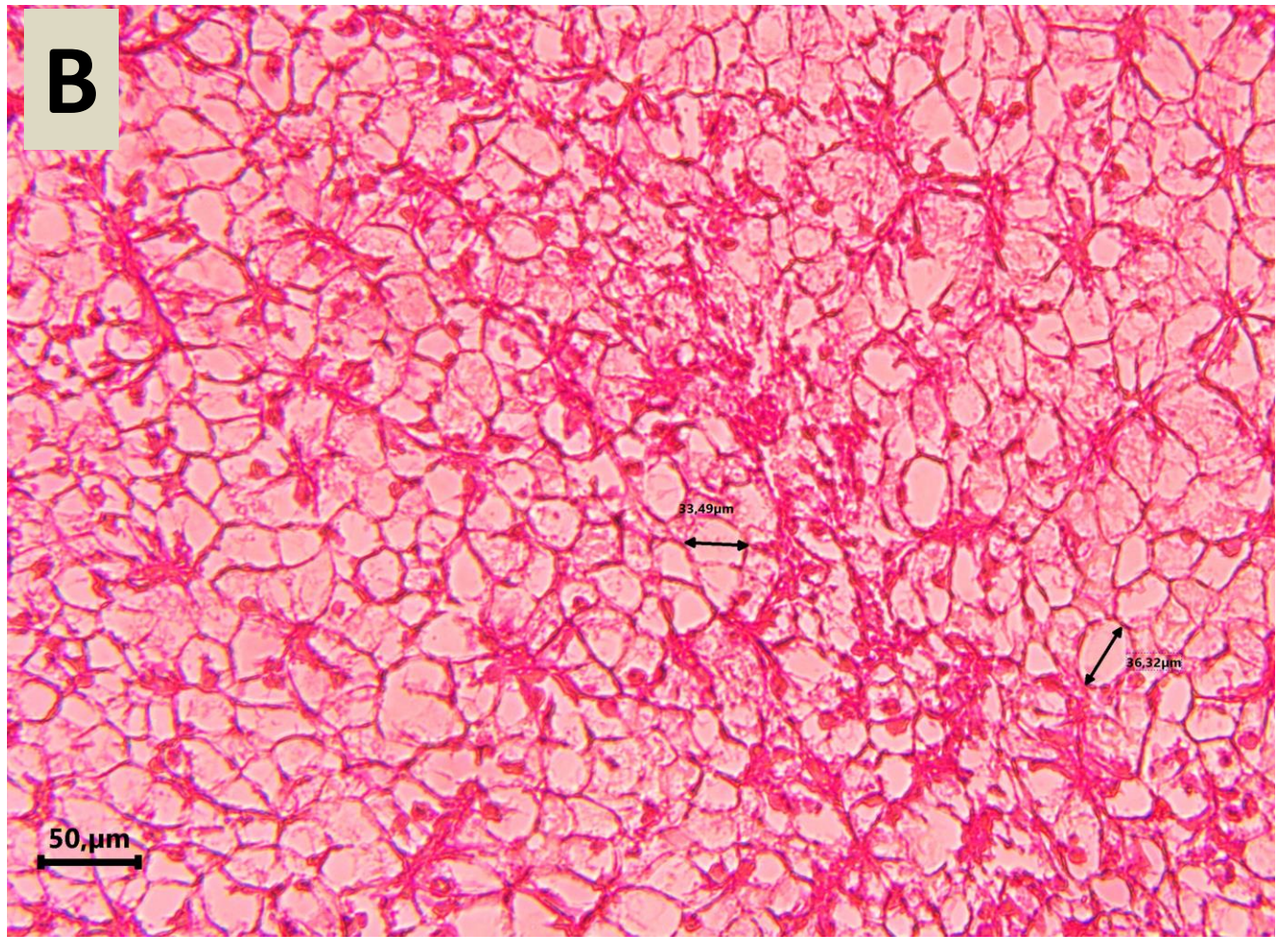
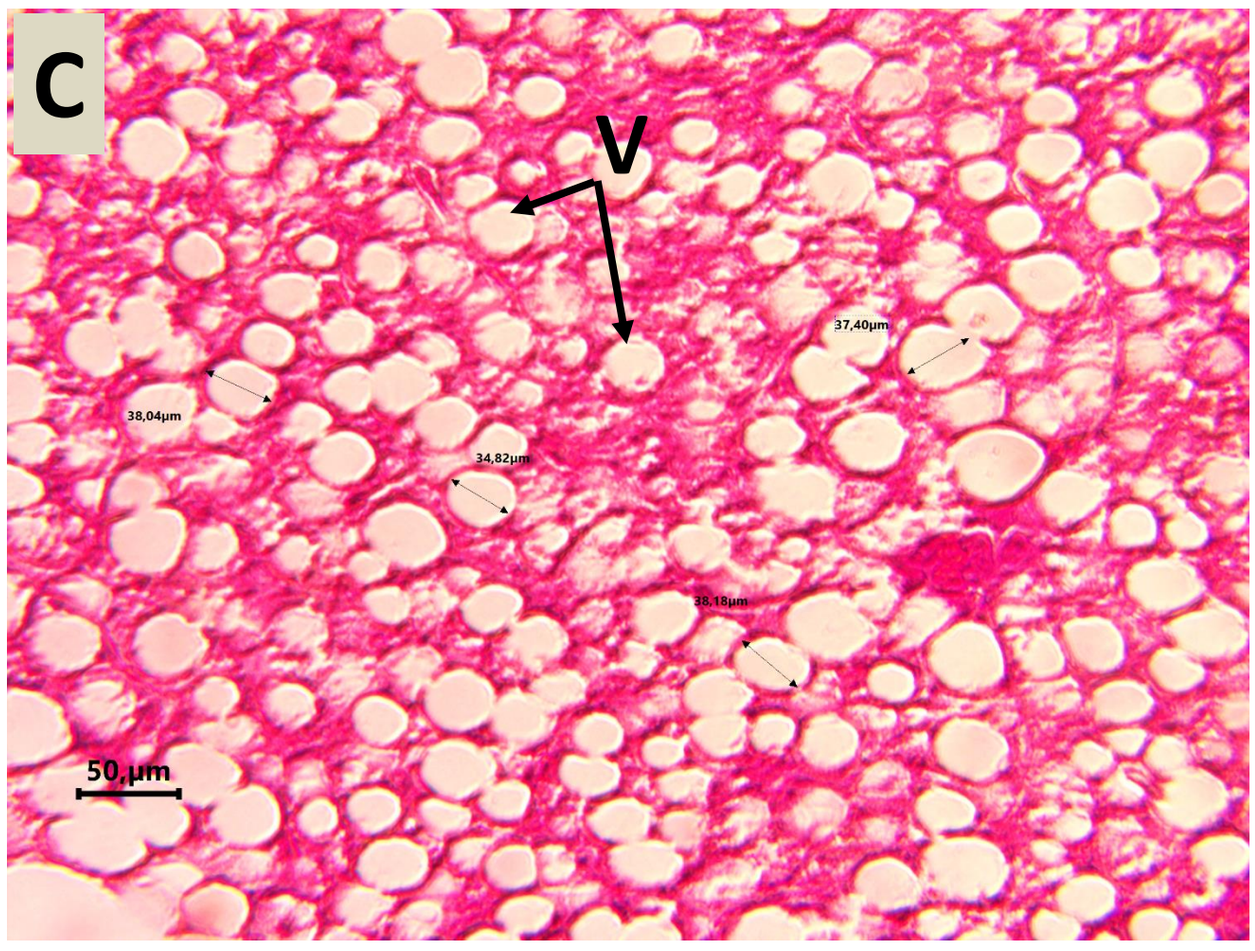

akt1
mtor
erk1/2
Relative mRNA expression
1
0.8
0.6
0.4
0.2
0
a
b
b
b
a
b
b
a
a
b
b
b
Control
AV 0,5%
AV2,5%
AV 5%
Control
AV 0,5%
AV2,5%
AV 5%
Control
AV 0,5%
AV2,5%
AV 5%

Figure.4


A
V
SV
HP
H
50,µm


B
33,49µm
36,32µm
50,µm


C
V
37,40µm
38,04µm
34,82µm
38,18µm
50,µm

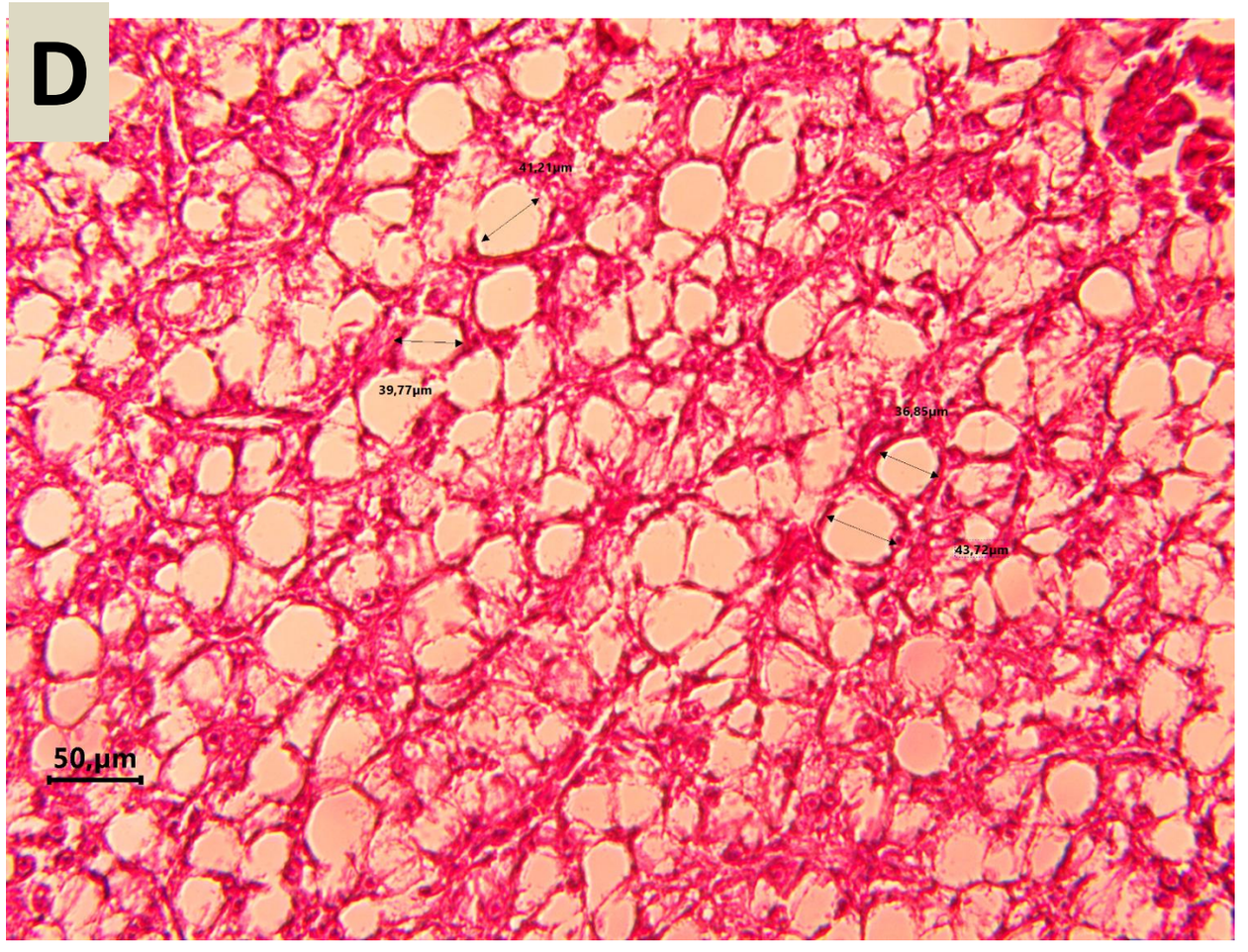

D
41,21µm
39,77µm
36,85µm
43,72µm
50,µm

Figure. 5

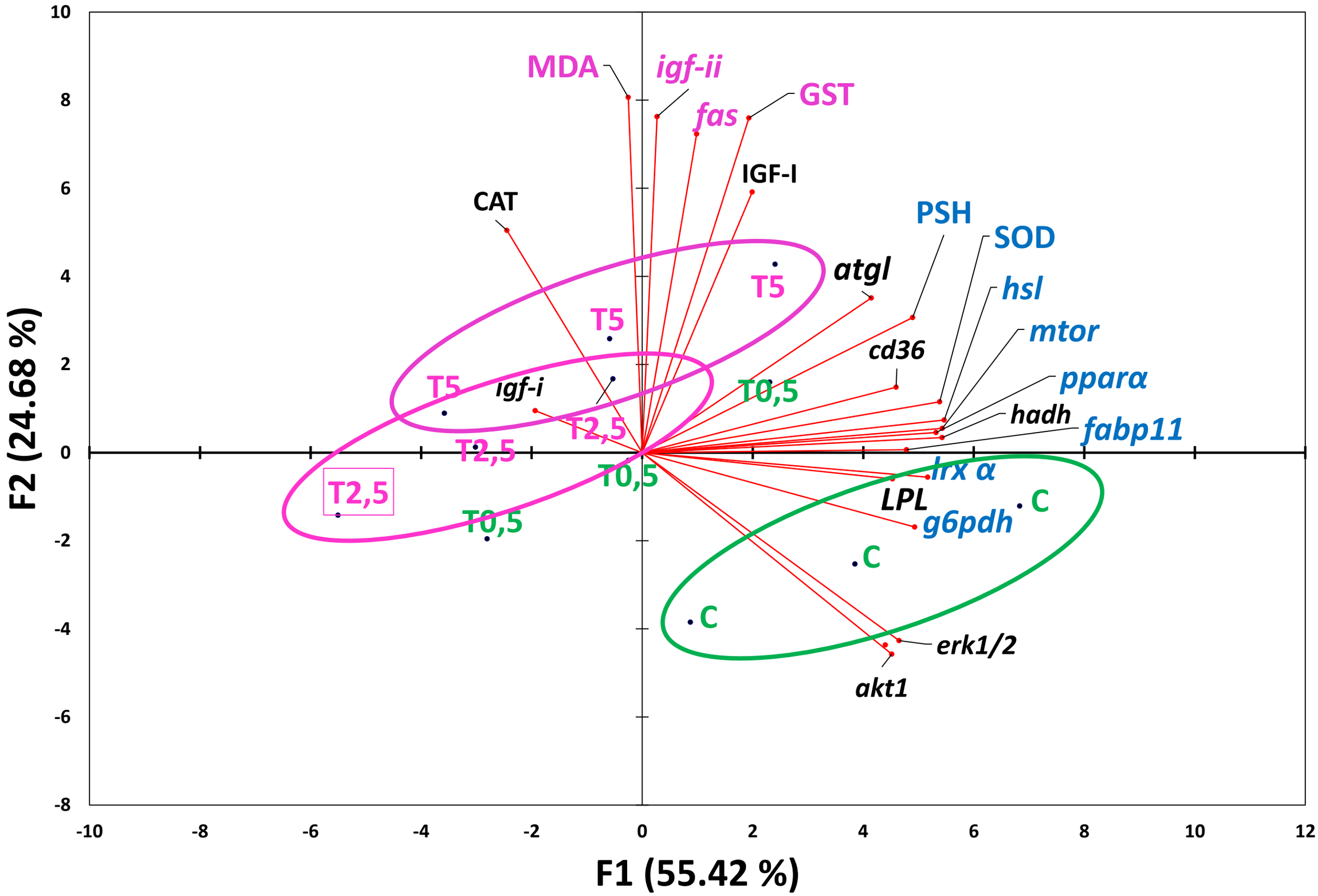

Biplot (axes F1 and F2: 80.10 %)
F2 (24.68 %)
F1 (55.42 %)
10
8
6
4
2
0
-2
-4
-6
-8
-10
-8
-6
-4
-2
0
2
4
6
8
10
12
MDA
igf-ii
fas
GST
IGF-I
CAT
PSH
SOD
atgl
hsl
mtor
cd36
ppara
hadh
fabp11
lrx α
LPL
g6pdh
erk1/2
akt1
igf-i
T5
T2,5
T0,5
C

Figure. 6

Table

Table 1: Composition of the basal diet

Ingredients	Proportion (%)
Protein	45.1
Lipids	19.1
Cellulose	3.5
Ashes	6.7
Total phosphorus	1.01
Calcium	1.75
Sodium	0.26
	Additives/ Vitamins (UI/Kg)
E672-Vit A	5000
E671-Vit D3	1000
	Trace elements (mg/kg)
E4-Cu (copper sulfate)	1
E5-Mn (manganese oxide)	8.3
E1 (iron sulphate)	40
E6-Zn (zinc oxide)	52
E2-1 (calcium iodate)	1.2

Table 2: Oligonucleotide primers used in the reverse transcription-qPCR analysis

Function classification	Gene	Sequence (5′-3′)	Tm °C	Accession number	References
Lipogenesis	***fas***	F: TGGCAGCATACACACAGACC R: CACACAGGGCTTCAGTTTCA	60	AM952430	Sánchez-Moya et al., 2020
	g6pdh	F: CAGAATGAAAGATGGGATGGAGTC R: TTCAGGTAAATGGCTTCGTTCG	60	AY754640	Bou et al., 2014
Lipolysis	***lpl***	F: GAGCACGCAGACAACCAGAA R: GGGGTAGATGTCGATGTCGC	60	AY495672	Sánchez-Moya et al., 2020
	atgl	F: GTGCTTCAGTCCTGGATGTCTTC R: AGCCTTGCAGGTCCATGTTGA	60	JX975711	Sánchez-Moya et al., 2020
	hsl	F:GCTTTGCTTCAGTTTACCACCATTTC R:GATGTAGCGACCCTTCTGGATGATGTG	60	EU254478	Vélez et al, 2019
β-oxidation	***ppar α***	F: TCTCTTCAGCCCACCATCCC R: ATCCCAGCGTGTCGTCTCC	62	AY590299	Bou et a., 2014
	hadh	F: GAACCTCAGCAACAAGCCAAGAG R: CTAAGAGGCGGTTGACAATGAATCC	60	JQ308829	Sánchez-Moya et al., 2020
	cd36	F: GTCGTGGCTCAAGTCTTCCA R:TTTCCCGTGGCCTGTATTCC	60	ERR12611_isotig20793	Balbuena-Pecino et al., 2019
FA transporters	***fabp11***	F: CATTTGAGGAGACCACCGCT R: ACTTGAGTTTGGTGGTACGCT	60	KM593130	Yan et al., 2015
FA homeostasis	***lxrα***	F: GCACTTCGCCTCCAGGACAAG R: CAGTCTTCACACAGCCACATCAGG	62	FJ502320	Cruz-Garcia et al., 2011
Growth	***igf-i***	F:ACAGAATGTAGGGACGGAGCGAATGGAC R: TTCGGACCATTGTTAGCCTCCTCTCTG	60	EF688016	Balbuena-Pecino et al., 2019
	igf-ii	F:TGGGATCGTAGAGGAGTGTTG R:CTGTAGAGAGGTGGCCGACA	60	AY996778	Balbuena-Pecino et al.,2019
Cell proliferation	***erk1/2***	F: GCTCTATGGCAAGGCTGAC R: TGCCTGGAAACGAGCTGTT	60	FM146973	Fernández et al., 2013
Anabolic processes	***mtor***	F: CAGACTGACGAGGATGCTGA R: AGTTGAGCAGCGGGTCATAG	60	MH594580	Rashidpour et al., 2019
Cell survival	***akt1***	F: GCTCACCCCACTCTTCAGAC R:AAATTGGGAAATGTGCTTGC	60	ERA047531	Vélez et al, 2019
Reference genes	***rps18***	F:GGGTGTTGGCAGACGTTAC R:CTTCTGCCTGTTGAGGAACCA	60	AM490061	Vélez et al, 2019
	ef1a	F:CTTCAACGCTCAGGTCATCAT R:GCACAGCGAAACGACCAAGGGGA	60	AF184170	Vélez et al, 2019

Table 3: Phytochemical composition and antioxidant activity of the methanolic crude extract of *Aloe vera* (AV) powder.

	Yield of methanolic extract (g extract/100 g DS)	**TPC** (mg GAE/g extract)	**TFC** (mg QE/g extract)	**CT** (µg cat/g extract)	**DPPH (IC50)** (µg/ml)
Lyophilized *Aloe vera (LAV)*	2.029	87.29*±3.54	52.27*±0.89	20.72*±0.72	895.25*±10.27
Dried *Aloe vera (DAV)*	2.923	120.75*±4.40	83.67*±2.92	32.68*±1.01	506.14*±9.23

Results were expressed as mean ± SEM n = 3. TPC: Total phenolic compounds; TFC: total flavonoid content; CT: condensed tannins; Gallic acid equivalent (GAE) per gram of extract; DS dried sample; mg quercetin equivalent (QE) per gram of extract; µg Catechin equivalent g extract (cat)

*means that lyophilized *Aloe vera* (LAV) and dried *Aloe vera* (DAV) are significantly different (0<0.05)

Table 4: Retention time (TR), molecular ions mass and UVmax of *Aloe vera* (AV) phenolic compounds identified by HPLC-DAD-MS.

Peak	TR (min)	λmax (nm)	[M-H]-	Identification
1	12.165	296	393	Aloeresin B (= Aloesin)
2	14.89	294	539	Aloeresin A
3	24.44	299	447	8-O-methyl-7-hydroxyaloin B
4	24.686	295	447	8-O-methyl-7-hydroxyaloin A
5	25.331	301	433	7-hydroxyaloin A
6	25.93	299	433	7-hydroxyaloin B
8	27.378	299	555	Isoalceresin D
9	31.831	268, 295, 354	417	Aloin A (=barbaloin)
10	33.385	268, 295, 355	417	Aloin B (=Isobarbaloin)

Table 5: Growth performance of fish fed with diets containing graded levels of *Aloe vera* (AV) powder for 8 weeks[1].

	Dietary *Aloe vera* (%) (g/100 g dry matter)			
	Control	**AV 0.5%**	**AV 2.5%**	**AV 5%**
Growth parameters	**Mean ± SEM**	**Mean ± SEM**	**Mean ± SEM**	**Mean ± SEM**
Initial weight (g)	142.13±0.92	139.48±3.94	138.35±1.45	143.98±0.73
Final weight (g)	244.41±3.27	244.74±4.35	228.49±5.46	239.27±0.53
Length (cm)	24.35±0.02	23.94±0.07	22.97±0.73	24.22±0.16
WG[2]	102.27	105.26	90.13	95.28
WGR[3]	71.99±3.04	75.72±5.47	65.10±2.50	66.19±1.20
SGR[4]	0.90±0.02	0.95±0.05	0.83±0,02	0.84±0.01
K factor[5]	1.69±0.01	1.78±0.01	1.90±0.14	1.68±0.03
IHS[6]	1.76±0.02	1.64±0.06	1.87±0,10	1.61±0.13
Survival rate[7]	100±0.00	100±0.00	91.66±8.33	91.40±0.43
FI[8]	255.07±2.17	251.02±7.05	248.95±2.54	259.17±1.33
FER[9]	0.40±0.01	0.42±0.03	0.36±0.01	0.36±0.00

[1]Data are mean value of triplicates ± SEM. Means without letter are not significantly different according to Duncan's test ($p<0.05$)

[2]WG (g): weight gain =W2-W2

[3]WGR (%): weight gain rate =100*(final total weight (g) – initial total weight (g) / initial total weight (g).

[4]SGR (%/day^{-1}): Specific growth rate = 100* (Ln final individual weight (g) – Ln initial individual weight / numbers of days).

[5]K factor (%): Fulton condition factor = 100*(final individual weight (g) / final individual length^3.

[6]IHS (%): Hepatosomatic index (%) = 100* liver weight (g) of final individual fish / final individual weight (g).

[7]Survival rate (%): 100* (final number of fish) / (initial number of fish)

[8]FI (g/fish): Feed intake= dry feed intake /number of fish.

[9]FER (g/g): Feed efficiency ratio= WG (g)/ FI (g)

Table 6: Number and diameter of lipid vacuoles in the liver of gilthead sea bream (*Sparus aurata* L.) fed with increased *Aloe vera* (AV) supplemented diets for 8 weeks

	Treatment	Mean of vacuoles number	Standard deviation	Groups			
Mean of vacuoles number	Control	53	6,55	**A**			
	AV 0,5%	97,5	4,38		**B**		
	AV 2,5%	182	4,9			**C**	
	AV5%	140,5	9,72				**D**
Mean of diameters of lipid vacuoles	Control	17,81	0,78	**A**			
	AV 0,5%	19,87	0,38		**B**		
	AV2,5%	29,35	0,6			**C**	
	AV 5%	30,63	0,53			**C**	

Results are expressed as mean ± SD. Different capital letters indicated significant differences between treatments using Duncan test.

Table 7: Principal component analysis (PCA) of biochemical and molecular parameters responses in *Sparus aurata* fishes treated with different doses of *Aloe vera* (AV) for 8 weeks

		Principal components	
		PC1 (55,42%)	**PC2 (24,68)**
Biochemical parameters	GST	0,1190	**0,8187**
	CAT	0,1907	0,3610
	SOD	**0,9234**	0,0188
	MDA	0,0020	**0,9225**
	PSH	**0,7634**	0,1334
	IGF-I	0,1262	0,4963
Molecular parameters	*lpl*	0,6539	0,0051
	hsl	**0,9519**	0,0078
	atgl	0,5476	0,1747
	pparα	**0,9005**	0,0030
	hadh	**0,9385**	0,0016
	fas	0,0310	**0,7418**
	g6pdh	**0,7753**	0,0405
	cd36	0,6730	0,0312
	fabp11	**0,7286**	0,0001
	lrx α	**0,8508**	0,0044
	igf-i(2)	0,1195	0,0129
	igf-ii	0,0023	**0,8255**
	akt1	0,6504	0,2968
	mtor	**0,9398**	0,0043
	erk1/2	0,6885	0,2584

Table 8: ANOVA of the scores of the first two principal components among *Aloe vera* (AV) treatment groups, followed by Duncan test

variables	treatment	score means	groups	
PC1	C	3,8519	**A**	
	AV0.5%	-0,2492	**A**	**B**
	AV 5%	-0,5867	**A**	**B**
	AV 2,5%	-3,0160		**B**
PC2	AV 5%	2,5842	**A**	
	AV 2,5%	0,1254	**A**	**B**
	AV 0,5%	-0,1793	**A**	**B**
	C	-2,5303		**B**

Conflict of interest

The authors confirm that there are no conflicts of interest in this work.

Author Statement

The authors thank the participants who gave their time to the trial. Jamel JEBALI and Isabel NAVARRO designed, supervised the study and revise manuscript. Afef AMRI designed, performed experiments, analyzed data and co-wrote the paper. Zied BOURAOUI contributed to animal experiments, samples collection and analyzed data. Sara BALBUENA-PECINO and Encarni CAPILA contributed to molecular analyses and revise manuscript. Hamadi GUERBEJ carried out the rearing work. Karim HOSNI supervised the phytochemical analysis and revise manuscript. Tahar GHARRED and Zohra HAOUAS contributed to the histological and statistical analysis of the results. All authors analyzed the results and contributed to the manuscript writing, and approved the final version of the manuscript.

Dietary supplementation with *Aloe vera* induces hepatic steatosis and oxidative stress together with a disruption of cellular signaling pathways and lipid metabolism related genes' expression in gilthead sea bream (*Sparus aurata*)

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Abstract

This study aimed to assess the effects of dietary increasing concentrations of *Aloe vera* (AV) powder of 0.5%, 2.5% and 5% on the growth performance, hepatic oxidative status, histology, and lipid metabolism and cellular signaling pathways-related genes' expression in gilthead sea bream (*Sparus aurata*). The preliminary phytochemical analysis revealed the richness of the dried AV extract on total phenol content, total flavonoid content, and condensed tannins when compared to the lyophilized sample. The dried extract showed a good DPPH-radical scavenging activity and its profiling by HPLC-DAD-ESI-MS revealed the presence of anthraquinones namely aloin A, aloin B and their hydroxyl (7-hydroxyaloin A and 7-hydroxyaloin B) and methyl-hydroxy (8-O-methyl-7-hydroxyaloin A and 8-O-methyl-7-hydroxyaloin B) derivatives as well as aloeresin A and B. The AV supplementation in fish diet did not affect growth performance (WG, WGR, and SGR) and feed utilization (FI, FCR, FER), and HSI indexes. However, the hepatic insulin-like growth factors (IGF-I and II) levels were significantly enhanced. Genes' expression levels of enzymes or transcription factors involved in lipolysis (*lpl*, *hsl*, and *atgl*), beta-oxidation (*pparα*, *hadh*), fatty acid transporters (*cd36, fabp11*) and *lxrα* were significantly down-regulated by the two high concentrations of AV powder. In contrast, fatty acid synthase (*fas*), a key gene of lipogenesis was significantly up regulated by dietary AV 5% powder supplementation. The induction of *fas* together with the down-regulation of peroxisome proliferator-activated receptor (*pparα*) and hydroxyacyl-coenzyme A dehydrogenase (*hadh*) could explain the lipid accumulation resulting in hepatic steatosis, which was confirmed by histological analysis, since the diets at the two higher concentrations (AV 2.5% and AV 5%) induced a significant increase in the number and diameter of hepatic lipid vacuoles in a dose dependent manner. Moreover, the mRNA levels of protein kinase B named (*akt*), mammalian target of rapamycin (*mtor*) and extracellular regulated kinase (*erk1/2*) involved in cell survival and proliferation were decreased by all AV powder supplemented diets. AV 5% increased catalase and glutathione S transferase activities suggesting a cellular strategy to fight against reactive oxygen species (ROS) accumulation. In conclusion, dietary supplementation with AV 0.5% is recommended for gilthead sea bream feed formulation, as it stimulates the *igf-i* expression. However, higher levels of AV should be avoided as they might cause lipid metabolism disruption, oxidative stress and liver steatosis.

Key words: *Aloe vera*, *Sparus aurata*, lipid metabolism, oxidative stress, gene expression.

1. Introduction

During the last decades, interest in using medicinal plants as feed additives in aquaculture has escalated due to their richness in bioactive ingredients with health beneficial effects. They are incorporated in farmed fish diet as crude powder/extracts, active compounds or in association with a probiotic (Awad et al., 2017). The use of plant-enriched diets has been shown to improve growth performance, antioxidant activities and immune system in fish (Yousefi et al., 2020; Zemheri-Navruz et al., 2020). However, some plants may contain anti-nutritional compounds or toxic metabolites. Therefore, careful phytochemical examination of the plant and dosage is required before their use as feed additives in aquaculture (Reverter et al., 2020). Among medicinal plants, *Aloe vera* (AV) has received particular attention owing to its antioxidant, anti-obesity, hypo-glycemic, hepato-protective, and immuno-modulatory properties (Maan et al., 2018; Kumar et al., 2019). Earlier compositional investigations showed that AV contained essential minerals, vitamins, saponins, polysaccharides, phenolic compounds including anthraquinones/anthrones (aloe-emodin, anthranol, aloin A and B, emodin, etc.), organic acids, amino acids, and lipids, which make it an essential ingredient in different cosmetic, pharmaceutic and agrifood products (Radha et al., 2015; Mann et al., 2018). Phytosterols from AV were found to induce the overexpression of genes involved in fatty acid transport (*acsl1*), fatty acid oxidation (*acaa1a*, *acox1*, *cpt1a*, *cpt2*, *cyp4a10*, and *cyp4a14*), and retinoid X receptor (*rxr*) in obese mice (Pothuraju et al., 2015). However, the AV anthraquinones were shown to be responsible for the toxicity in primary rat hepatocytes and may be the main constituent responsible for liver injury (Liu et al., 2020). Of all the components of AV, aloin is the most toxic anthraquinone and it appears in two forms: aloin A and B (Kaparakou et al., 2021). Despite the evidence on the differential effects of AV components, there are limited data

on the potential effect of this plant on the transcription network that regulates the hepatic lipid metabolism in fish, specifically, in gilthead sea bream.

Lipid metabolism in fish is a complex phenomenon accrued in liver and other organs such as adipose tissue and muscle; it is widely regulated via the interaction between hormones, transcription factors, growth factors and the involvement of many enzymes in the lipogenic and lipolytic pathways (Bullón-Vela et al., 2018). Transcription factors including liver X receptor (*lxrα*) and peroxisome proliferator-activated receptors (*pparα*) are of particular importance in controlling lipid metabolism in fish (Bou et al., 2014; Wei et al., 2017). The former *lxr* factor is involved in cholesterol metabolism (Ayisi et al., 2018), while the latter *pparα* is implicated in the β-oxidation of fatty acid (FAs) originally produced though lipoprotein lipase-mediated hydrolysis of triglycerides and very low-density lipoproteins (Kidani et al., 2012; Weil et al., 2013). Therefore, a disturbance in that process resulted in a reduced capacity of utilization of free fatty acids (FFA) energy, and consequently, development of steatosis (Bullón-Vela et al., 2018). In general, metabolic disturbance are caused by the overproduction of reactive oxygen species (ROS) leading to oxidative stress (Bullón-Vela et al., 2018; Kim et al, 2020).

Gilthead sea bream (*Sparus aurata*) is considered one of the most important fish species farmed in the Mediterranean area including Tunisia using intensive farming system. Its production increases annually, and is estimated nearly 64,000 tones (Konstantinidis et al., 2021). In capture fisheries, growth performance of Sparus aurata is conditioned by numerous factors including temperature, feed composition and feeding strategies, among others (Mongile et al., 2014). To improve growth performance in farmed fish, feeding strategies based on replacement of fishmeal (FM) and fish oil (FO) have been reported (Xu et al., 2019). Furthermore, other feeding strategies including the supplementation with minerals (Dominguez

et al., 2017), FAs (Turkmen et al., 2020), vitamins (Lanyau-Domínguez et al., 2021), and plant extracts (Nhu et al., 2019) have been successfully assayed. Feeding these functional diets resulted in improved growth with a concomitant enhanced immunity and disease responses as well as increased appetite (Citarasu, 2010).

Prompted by these antecedents and considering the multiple properties of AV, we investigated the chemical composition of AV powder and assessed its potential effects on the growth performance of gilthead sea bream. A special attention was paid for the first time to the hepatic lipid metabolism and cellular signaling pathways related genes' expression, as well as antioxidant defense and liver histology under standard rearing conditions.

2. Material and methods

2.1. Plant sample

Leaves of AV were collected in the region of Teboulba (East-Central Tunisia; Latitude 35°64'86" (N); Longitude 10°97'48" (E)). After washing with tap water, they were cut off from their base and placed vertically for 2-4 h to remove the latex liquid containing anthraquinones (Zanuzzo et al., 2017). Thereafter, they were cut into small pieces and divided into 2 batches. The first batch was dried at 50°C in a forced-oven air for 12 h and powdered using a laboratory mill. The second batch was lyophilized for 72 h using a Christ Alpha 2-4 freeze drier (Osterode, Germany). The dried and lyophilized powder samples were stored at -20°C until processed.

2.2. Preparation of extract

The powdered raw material was extracted using methanol (1:20, w/v) for 24 h at room temperature under constant shaking using an orbital shaker. The combined supernatants were filtered through Wattman filter paper (0.22 µm) and concentrated under reduced pressure in a

Heidolph rotary evaporator (Schwabach, germany). The resulting dried methanol extract was subsequently assayed for its phytochemical analyses and biological activities.

2.3. Phytochemical analyses

All phytochemical measurements were performed in triplicate for each analysis.

2.3.1. Determination of total phenolic content (TPC)

The TPC was determined using the Folin-Ciocalteu (FC) reagent as described by Singleton & Rossi (1965). Briefly, a 500 µL aliquot of sample extract dissolved in methanol or the standard gallic acid (GA) was mixed with 2.5 mL of 10-fold diluted FC reagent. After 4 min, 2 mL of 7.5% NaCO3 was added and the mixture was left to stand for 2 h in the dark at room temperature. The absorbance was then measured at 760 nm and the TPC were expressed as mg of gallic acid equivalents per gram extract (mg GAE/g extract).

2.3.2. Determination of total flavonoid content (TFC)

For the determination of TFC, the colorimetric method with AlCl3 was used with modifications (Dehpour et al., 2009). Briefly, a 500 µL aliquot of sample extract or the standard quercetin (Q) was mixed with 1.5 mL methanol, 100 µL 10% AlCl3, 100 µL 1 M potassium acetate, and 2.8 mL distilled water. After 30 min incubation at room temperature, the absorbance was measured at 415 nm and the results were expressed as mg quercetin equivalents per gram extract (mg QE/g extract).

2.3.3. Determination of condensed tannins (CT)

The CT was estimated using the vanillin method as described by Broadhurst and Jons (1978). A 500 µL aliquot of sample extract or the standard catechin (C) was mixed with 3 mL 4% vanillin (in methanol) and 1.5 mL concentrated HCl. After 2 h incubation at room temperature,

the absorbance was measured at 500 nm and the results were expressed as µg catechin equivalents per gram extract (µg CE/g extract).

2.3.4. Characterization of phenolic compounds by HPLC-DAD-ESI-MS

The HPLC-DAD-ESI-MS analysis was performed on an Agilent 1100 series HPLC systems equipped with a photodiode array detector (PDA), a triple quadrupole mass spectrometer type Micromass Autospec UltimaPt (Kelso, UK) and an electrospray source (ESI) ion source operating in negative mode. The column was a C18 reversed-phase Superspher®100 (12.5 cm × 2 mm id; 4 µm, Agilent Technologies, Rising Sun, MD) and its temperature was maintained at 45°C. The mobile phase consisted of a combination of A (0.1% acetic acid in water) and B (acetonitrile) with a flow rate of 0.25 mL/min. The solvent gradient consisted of a multi-step linear gradient: from 0 to 2% B in 5 min, 88% B at 75 min, and decreasing to 2% B in 90 min. The Uv-Vis spectra were recorded from 200 to 800 nm, and ions in the m/z range of 100-1000 were detected using a scan time of 1 s. The ESI source was conducted under the following operating conditions: capillary voltage, 3.2 kV; cone voltage, 115 V; probe temperature, 350° C and ion source temperature, 110° C (Mejri et al., 2018). Data acquisition and analysis were performed with a Masslynx software version 4.0. Because of the lack of authentic standards, the tentative identification of compounds was carried out comparison of their UV and mass spectra, as well as their fragmentation pattern with literature data (Koyama et al., 2009; Fanali et al., 2016; El Sayed et al., 2016)

2.4. DPPH (2, 2'-diphenyl-1-picrylhydrazyl) radical scavenging activity

The DPPH-free radical scavenging activity of the methanol extract of AV was determined according to the method of Binsan et al. (2008). One milliliter sample extract at various concentrations (from 0.01 to 2 mg mL-1) was added to 2 mL of 0.1 mM DPPH methanolic

solution. The mixture was Vortexed (Stuart SA8, Thermo Fisher Scientific Inc., Bordeaux, France) vigorously and allowed to stand for 30 min in the dark. Thereafter, the absorbance was measured at 517 nm against the control blank containing methanol and performed in triplicate. The percent inhibition of DPPH-free radicals was calculated as follows:

$$\% \text{ Inhibition} = [(A \text{ blank} - A \text{ sample})/A \text{ sample}] \times 100$$

Where A blank is the absorbance of the control and Asample is the absorbance of extract sample.

The DPPH-radical scavenging activities were expressed as EC50 that represents the sample concentration required to reduce the initial DPPH concentration by 50%.

2.5. *In vivo* experiments

2.5.1. Preparation of experimental diets

Four experimental diets were prepared; a control diet without AV powder and three more diets were supplemented with graded levels of AV powder. The composition of the commercial diet is shown in table 1 [crude protein (45.1%), fat (19.1%), ash (6.7%) and cellulose (3.5%)] (Skretting, Spain, 4 mm). This diet was ground into powder; thereafter, the crude powder corresponding to 0.5 %, 2.5% and 5% of AV was weighed and they were blended together manually about 10-15 min. Then, distilled water was added bite by bite until a stiff dough resulted as required. The control diet underwent the same steps but only with water. The paste of each diet was then separately passed through a mincer with 4 mm die resulting in strands, which were gently broken into fresh pellets, air-dried at ambient temperature for 3 days (Gabriel et al., 2015; Amri et al., 2020). The diet was prepared twice a week.

2.5.2. Fish acclimatization and experimental design

A total of 144 healthy gilthead sea bream fish were obtained from the National Institute of marine Sciences and Technologies of Monastir (INSTM, Monastir-Tunisia). Fish were held in 12 tanks (1000 L, 12 fish/tank) and initially fed with a commercial diet (Skretting, Spain, 4mm) twice a day (9:00 AM and 03:00 PM) during one week for acclimatization. The physicochemical proprieties of seawater were monitored every 10 days during the trial. Temperature, 22±1°C; salinity, 37±2 mg/l; oxygen, (>=3.5 mg/l) and photoperiod (12 h light/12 h dark). The open circuit system (flow-through) was adopted for the seawater continuous changing at the flow rate of 100%/h and with continuous aeration.

After the adaptation of fish to the experimental system they were anesthetized with 2-phenoxyethanol (0.2 mL L-1; Sigma). For each fish, the initial body weight and length were measured to calculate the biometrics parameters. Four groups of fish were installed and received the experimental diets as following: group 1 fed with control diet (without AV), group 2 fed with A.V 0.5% supplemented diet (5 g/kg feed), group 3 received dietary AV 2.5% (25 g/kg feed) and finally group 4 dieted with A.V 5% (50 g/kg feed). The experiments were done in triplicate (36 fish/group). Each fish group was fed with the corresponding diet by hand for 60 days, 6 days a week, 2 times a day (9:00 AM and 03:00 PM) until satiation.

2.5.3. Sampling

After 8 weeks feeding period, fish were fasted 24 h before sampling to avoid regurgitation of food. Thereafter, fish were euthanized by a blow to the head under anesthesia by phenoxyethanol (0.2 ml/l) diluted in seawater (Sánchez-Nuño et al., 2018). The fish were weighted, measured and dissected, and liver was gently rinsed in phosphate buffered saline (PBS), blotted dry and then divided into 3 parts. The first part immediately placed in RNA later

(25 mM sodium , pH 5,2; 1:10 w/v) left at 4°C overnight and subsequently stored at -80°C until gene expression analysis (6 samples/group). The second part of the liver was fixed in phosphate buffered formalin 10%, pH =7.4 for 24 h and dehydrated in ethanol (70%) for histological examination (9 samples/group) and the last part of the liver was stored at -80°C for biochemical analysis (10 samples/group).

2.5.4. Fish growth, survival and feed utilization performance

The individual body weight and length were measured at the beginning and at the end of the AV feeding. Growth parameters and survival rate were measured according to Amri et al. (2020) and as follows:

Weight gain; WG (g) = final mean weight- initial mean weight.

Weight gain rate; WGR (%): weight gain rate =100*(final total weight (g) – initial total weight (g) / initial total weight (g).

Specific growth rate; SGR (%/day-1): Specific growth rate = 100* (Ln final individual weight (g) – Ln initial individual weight / numbers of days).

Condition factor; CF (%) = 100*(final individual weight (g) / final individual length3.

Hepatosomatic index; HSI (%) = 100* liver weight (g) of final individual fish / final individual weight (g).

Feed intake; FI (g/fish): dry feed intake /number of fish.

Feed efficiency ratio; FER (g/g): = WG (g)/ FI (g).

Survival rate (%): 100* (final number of fish) / (initial number of fish)

2.6. Molecular analysis

2.6.1. RNA extraction and cDNA synthesis

As previously described by Balbuena-Pecino et al. (2019), total RNA was extracted from 40 mg of liver with 1 ml of Tri Reagent solution following the manufacturer's recommendations (Applied Biosystem, Alcobendas, Spain). A NanoDrop 2000 (Thermo scientific, Alcobendas, Spain) was used to determine total concentration and purity. Analysis by 1% agarose gel electrophoresis stained with SYBR- safe DNA gel stain (Life Technologies, Alcobendas, Spain) was used to check the integrity of samples. To remove all genomic DNA, total RNA (1000 ng) were treated with DNase I (Life Technologies).Thereafter, RNA was reverse transcribed with the transcriptor first strand cDNA synthesis kit (Roche, Sant Cugat del Valles, Spain).

2.6.2. Real time quantitative PCR (qPCR) analyses

The mRNA transcript levels analysis of the target genes plus two reference genes were examined in CFX 384™ real time system (Bio Rad , El prat de Lobregat , Spain) following the procedures previously described by Balbuena-Pecino et al. (2019). All the analysis were performed in triplicate wells using 384 well plates with 2.5 µl of iTaq universal SYBR Green supermix (Bio-Rad), 250 nM final concentration of forward and reverse primers (Table 2) and 1 µl of diluted cDNA for each sample, in a final volume of 5 µl. The expression level of each analyzed target gene was calculated using the Pfaffl method (Pfaffl, 2001) relative to the geometric mean of the two reference genes (rps18) and (ef1a) as they were both constitutively expressed and not affected by the treatments. Both, relative expression calculations and reference genes stability determined by the geNorm algorithm, were done using the implemented Bio Rad CFX manager3.1.software.

2.7. Biochemical analysis

2.7.1.. Liver homogenization and protein quantification

All steps were carried out at 4 °C. Liver samples were homogenized in ice-cold phosphate buffer (100 mmol/L; pH 7.5) using an Ultra-turrax homogenizer. The homogenates were centrifuged at 10.000×g at 4°C for 20 min and the supernatants were then used the estimation of TBARS, PSH, and antioxidant enzymes activity. The total protein level was determined at 595 nm, using bovine serum albumin (BSA) as a standard (Bradford 1976).

2.7.2. Thiobarbituric reactive substances level determination (TBARS)

Liver TBARS, markers of lipid peroxidation were measured by the colorimetric assay of Buege and Aust (1978) using 1,1,3,3-treaethyloxypropane as a standard. The mixture containing liver extract, 15% TCA, 0.375% TBA, and 1 N HCl was heated for 20 min at 95°C (water bath) and then centrifuged. The absorbance of the supernatant was measured at 512 nm and the results were expressed as μmoles/mg protein.

2.7.3. Protein sulfhydryl level determination (PSH)

Liver protein sulfhydryl (PSH) level was determined by the subtraction of non-protein sulfhydryl (NPSH) content from total sulfhydryl content (TSH) (Absorbance1- Absorbance2) (Sedlak and Lindsay, 1968). The concentration of TSH was determined using DTNB (0.01 M) and the absorbance was measured at 412 nm (A1). After precipitation of the sulfhydryl proteins (PSH) by10% TCA, the clear supernatant containing NPSH was treated with DTNB (0.01 M), which was reduced to yellow TNB (5-thiobis (2 nitrobenzoic acid)) and the absorbance was measured at 412 nm (A2). PSH content was expressed as μg per mg of protein.

2.7.4. Antioxidant enzyme activity

Liver catalase (CAT) activity was spectrophotometrically determined by monitoring the disappearance of hydrogen peroxide (H2O2) at 240nm (Claiborne, 1985). Briefly, 10 μL of liver supernatant was mixed with 890 μL phosphate buffer (100 mM, pH 7.5),and 100 μL of

500 mM H_2O_2 nd The absorbance at 240 nm was measured and the CAT activity was then determined and expressed as μmoles H_2O_2/min/mg protein.

Superoxide dismutase (SOD) activity was determined based on its ability to inhibit the auto-oxidation of pyrogallol (Marklund and Marklund 1974). Briefly, 200 μL of a cold mixture ethanol: chloroform (1:1, v/v) was added to 250 μL of liver supernatant was and centrifuged at 2500×g for 25 min at 4 °C. The absorbance was immediately measured at 420 nm in the presence of pyrogallol. SOD activity was expressed as units per milligram of protein (U/mg of protein). One unit represents the amount of SOD, which inhibits 50% of pyrogallol autoxidation.

For GST activity, the method of Habig et al. (1974) using 1-chloro-2,4- dinitrobenzene as substrate and glutathione (1 and 4 mmol/L final concentration, respectively) in 100 mmol/L sodium phosphate buffer (pH 7.4) was used. The absorbance was measured at 280nm and the GST activity was expressed as micromoles produced per minute per milligram protein. (μmol/min/g of protein).

All antioxidant enzyme activity assays were performed in conditions of linearity with respect to incubation time.

2.7.5. Insulin-like growth factor-1 levels determination (IGF-I)

Insulin-like growth factor-1 levels were measured in liver using a commercially available ELISA fish kit (m/r IGF-I ELISA; CUSABIO Bio., China). Liver samples were homogenized in 0.1 M PBS (pH 7) (1:10, w/v) and centrifuged at 12000×g for 30 min at 4°C.The supernatants were collected and subsequently assayed for IGF-I following the manufacturer's instructions.

2.8. Histological study

Three fish liver from each tank (nine livers per group) were used for the histological analysis. Three sections from each liver were performed for the analysis of the lipid vacuoles. Samples fixed in 10% buffered formalin were dehydrated through graded alcohol, then xylene and finally embedded in paraffin wax. The paraffin blocks were sectioned at 3 µm, stained with hematoxylin and eosin (H & E) and examined using a light microscope at 400× magnification.

2.9. Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM). In histomorphological evaluation, the standard deviation (SD) was used. Normality and homogeneity of variance were checked using the Shapiro–Wilk and the Levene's tests, respectively. One-way analysis of variance (ANOVA) followed by Duncan's post- hoc multiple range test was used to compare group means at a significance level of $p < 0.05$ and student t-test was used for phytochemical analysis in order to compare the lyophilized and dried AV extracts (LAV and DAV) ($p<0.05$). PCA analysis was used to simplify the data sets, and to determine the correlations between molecular and biochemical parameters and to define the main contributors to the total variance. Statistical analyses were performed using SPSS Statistics v.22 (IBM, Armonk, USA) and using XLSTAT 2.5 (Addinsoft New York, NY).

3. Results

3.1. Chemical composition of the AV powder

The mean values and SD of the yield of methanolic extract, TPC, TFC, CT, and DPPH of the lyophilized and dried AV powder are presented in Table 3. Compared to the lyophilized sample, the dried powder showed the highest extract yield, TPC, TFC and CT contents, and exhibited the strongest DPPH-radical scavenging activity. For this reason, the dried powder was selected

to elucidate its phenolic profile and to investigate its possible effect on lipid metabolism related genes and oxidative stress defense of the sea bream (*Sparus aurata*) under standards rearing conditions.

Tentative identification of phenolic compounds of AV dried powder was made based on their mass and UV spectra (Table 4). Overall, the methanolic extract enclosed 3 chromones (Aloeresin A, Aloeresin B and Isoalceresin D) and 6 anthraquinones (Aloin A, Aloin B, 7-hydroxyaloin A, 7-hydroxyaloin B, 8-O-methyl-7-hydroxyaloin A, 8-O-methyl-7-hydroxyaloin B).

3. 2. Effect of AV powder on growth performance

As shown in table 5, the diets supplemented with increasing concentrations of AV powder had no significant effects on the growth parameters. Slight but no significant decreases in WGR and SGR were observed in fish fed with AV 2.5 and 5% supplemented diets. Feed intake did not significantly differ between all groups. However, a slight decrease in feed efficiency was observed in fish fed with 2.5 and 5% AV supplemented diets. The HSI remained unchanged between control and treated groups.

3.3. Effect of AV powder on oxidative stress parameters

The AV powder addition in fish diets at levels of 0.5, 2.5 and 5% dose dependently enhanced ($p<0.05$) the TBARS levels in treated groups compared to control one (Figure. 1A).

Concerning antioxidant enzymes, the results indicated that the AV 2.5% powder supplemented diet significantly diminished the SOD activity by 66.20% when compared to the control (Figure. 1B). The level of hepatic PSH was decreased significantly in fish fed with AV 2.5% supplemented diet when compared to the control and to the other fish groups fed with AV 0.5% and AV 5 % supplemented diets (Figure. 1C).

The hepatic CAT activity was enhanced in fish groups fed with AV 2.5% and AV 5% supplemented diets. The increase was about 2.41 and 1.81 times respectively (Figure. 1D). Regarding the GST, its activity was significantly increased in the fish fed AV 5% supplemented diet (Figure. 1E).

3.4. Effect of AV powder on the IGF-I levels and on the hepatic IGF-I and IGF-II genes expression

The effects of AV supplemented diets on hepatic IGF-I levels and on the gene expression of IGF I and IGF-II involved in the fish growth performance are presented **in Figure. 2.** The IGF-I levels were increased significantly in fish fed with AV 2.5% supplemented diet (Figure. 2A). The molecular analysis of mRNA expression of igf-I gene showed a significant enhancement in fish fed with 0.5%, and 2.5% supplemented diets about 2.34 fold and 2.25 fold respectively (Figure. 2B). Whereas, a 2.6 fold increase of igf-II gene expression was observed in the group treated with the highest concentration of dietary AV 5% as compared to the relative control (Figure. 2B).

3.5. Effect of AV powder on hepatic lipid metabolism-related genes expression

Figure 3 shows the relative changes of hepatic mRNA expression in lipid metabolism-related genes after 8 weeks of feeding with dietary increasing levels of AV powder. The lipogenic gene *fas* expression remained unaltered by dietary AV 0.5% and AV 2.5%; however, it was enhanced in fish fed with diet supplemented with the highest concentration of AV 5% by 5.5 times when compared to the relative control (Figure. 3A). The expression of lipolytic genes (*hsl*) and (*atgl*) and *g6pdh* were significantly reduced in fish fed AV 2.5% supplemented diet (Figure 2A, B). Regarding the (*lpl*) mRNA level, it was significantly downregulated in the fish fed with AV 5% supplemented diet by 47.36% when compared to the relative control. The expression of β-

oxidation related genes was affected in fish fed with A.V 2.5% and 5% but a significant decrease was observed only in the fish fed with AV 2.5% supplemented diet compared to all groups (Figure. 3C). Moreover, *ppar-α* and *hadh* genes expression, was significantly decreased by 53.86% and 58.20%, respectively in fish fed with AV 2.5% when compared to the relative controls. Among fatty acid transporters, *cd36*, which is a class B scavenger receptor that binds many ligands, including fatty acids, and phospholipids, showed a significant downregulation in fish fed with the AV 2.5% supplemented diet. The fatty acid-binding proteins (*fabp*) are a group of fatty acid-trafficking molecules that affect cellular functions. Compared to the control group (Figure 3), the *fabp11* gene expression was reduced by 69.1, 66.8 and 51.3% in fish fed with diets supplemented with 0.5, 2.5 and 5% AV, respectively. The *lxrα*, which is implicated in cholesterol and fatty acid homeostasis was downregulated by 61,6, 58.5 % and 43.6 % in groups treated with AV 0.5, 2.5 and 5%, respectively (Figure 3E).

3.6. Effect of AV on the expression of hepatic genes linked to cellular signaling pathways

In order to investigate whether dietary AV powder influences the expression of genes involved in cell signaling pathways, we analyzed the serine-threonine protein kinase (*akt1*), target of rapamycin (*mtor*) and extracellular signal-regulated protein kinase (erk1/2) mRNA levels. The results showed that the akt11 gene expression was dose dependently decreased by 38.18, 60.51 and 74.10% in fish fed with 0.5, 2.5 and 5% A.V supplemented diets respectively when compared to the relative control group (Figure. 4).

Furthermore, *mtor* mRNA levels were reduced in treated fish groups, although the decrease was only significant in the fish fed with AV 0.5% and AV 2.5% supplemented diets. Regarding *erk1/2* gene, its expression showed a significant decrease in all fish groups fed with experimental diets compared to the control group (Figure. 4). The reduction was evaluated

about 32.07, 68.92 and 64.23% in fish fed with AV 0.5%, AV 2.5% and AV 5% supplemented diets respectively when compared to the relative control group.

3.7. Histological evaluation

Examination of the liver tissue of the control fish showed the characteristic morphology, which consists of hepatocytes (H), sinusoid veins (SV) and an exocrine pancreas or hepatopancreas (HP). The hepatic parenchyma was made up of hepatocytes (H) which were polyhedral cells with a large centrally located nucleus with a few lipid vacuoles (V) (Figure. 5A).

The quantitative histological analysis showed that, in the fish fed with diets supplemented with increasing doses of AV powder for 60 days, there was a significant increase in the number and diameter of the lipid vacuoles compared to the control group (Table 6). Indeed, the number of vacuoles was increased by 1.83, 3.25 and 2.65 times in fish fed with diet supplemented with AV 0.5 %, AV 2.5% and AV 5%, respectively. The group of fish fed with the diet supplemented with AV 2.5% had the highest number of lipid vacuoles. Moreover, the diameter of lipid vacuoles increased by 1.11, 1.64 and 1.71 times in fish fed AV 0.5%, 2.5% and 5%, respectively (Table 6).

3.8. PCA of the biochemical and molecular responses of fish to the AV supplemented diets

PCA is a very useful tool to investigate the effect of the dietary increasing concentrations of A.V. on the molecular and biochemical parameters responses of treated fishes.

PCA is carried out on biochemical (GST, MDA, PSH, IGF-I, CAT and SOD) and molecular parameters (*lpl, atgl, hsl, fas, g6pdh, cd36, fabp11, ppar, hadh, lxr, akt1, erk1 / 2, mtor, igf-i, igf-ii*,) of control and treated fish fed with diets supplemented with AV 0.5%, AV2.5% and AV 5%.

The total variation of the two principal components (CP1 and CP2) is 80.10%. The first PC represented 55.42% of the total variance and was positively correlated with the PSH, *hsl, pparα, hadh, g6pdh, fabp11, lxrα* and *mtor* expression. The PC1 axis clearly separated the untreated control group from the group fed with diet supplemented with AV 2.5% AV.

The PC2 which accounted for 24.68% of the total variance, was positively correlated with the GST, MDA, *fas and igf-ii* (Table 7), and allowed the separation of control group from that fed with diet supplemented with AV 5% (Table 8).

4. Discussion

The use of plant supplements to improve growth, physiological performance and mitigate lipid deposition in fish has recently attracted much attention as an inexpensive, sustainable and effective alternative in aquaculture (Van Hai, 2015; Reverter et al., 2021). However, due to the inherent chemical composition, some plants can exhibit distinct levels of bioactivity and sometimes toxicity; therefore, their detailed phytochemical analysis and the search for optimal concentrations are important to assess their potential for the aquaculture sector (Reverter et al., 2021, Hoseini et al., 2021). Among these plants, AV was widely used as a functional and therapeutic food for both humans and mammals due to its anti-obesity, hypo-glycemic, immune-modulatory and antioxidant properties (Kumar et al., 2019; Maan et al., 2018; Gabriel et al., 2015). Nevertheless, several adverse effects have been explored, which might be relevant to the tested parts of the plant, the time of harvest, the climatic conditions, the extraction or purification processes, the indicated concentration and/or the tested animal system (Tong et al., 2021).

The HPLC-DAD-ESI-MS profiling of the methanol extract of the dried AV, allowed the identification of 9 compounds including 3 chromones (Aloeresin A, Aloeresin B and

Isoalceresin D) and 6 anthraquinones (Aloin A, Aloin B, 7-hydroxyaloin A, 7-hydroxyaloin B, 8-O-methyl-7-hydroxyaloin A, 8-O-methyl-7-hydroxyaloin B). These compounds have already been reported as distinctive phenolic compounds in different Aloe species (Añibarro-Ortega et al., 2019; Li et al., 2018) and most of them are credited with a long list of biological activities including antioxidant, anti-bacterial, antimycoplasmic and anti-cancer activities, among others (López et al., 2013).

In the current study, AV supplementation had no beneficial effects on fish growth (WG, WGR, and SGR) and feed utilization (FI, FCR, FER) indexes. Growth-promoting effects of herbal supplementation depends on the fish species, the type, and the concentration of the used material (Hoseini et al., 2021). For example, Mehrabi et al. (2019) showed that dietary supplementation of AV in the diet of rainbow trout at 5, 10 and 15% for 8 weeks significantly improved their growth performance. However, Gabriel et al. (2015) demonstrated that the inclusion of the plant at 0.5% and 2% improved growth indexes in GIFT-strain tilapia fish, while the higher dietary AV concentration (4%) did not affect these parameters. Growth is controlled being the most influential genes those of insulin-like growth factors (IGF-II and I) as they are the core of the hypothalamic–pituitary–somatotropic axis (HPS) together with the growth hormone (GH) (Triantaphyllopoulos et al., 2020). The effects of dietary conditions on insulin growth factors gene expression was reported to be a great potential to optimize fish growth rate but in the present study, the increase in IGF-I and IGF-II gene's expression and levels by AV diets was not associated with the enhancement of gilthead sea bream growth indexes. A possibility to explain these differences, it could be that although hepatic IGFs are enhanced by AV, the experimental period may not sufficient to show a significant induction of somatic growth by these peptides and the growth rates of fish of the size used in this experiment

are slower than that of small juveniles (Myrick and Cech 2000). Furthermore, the endocrine mechanisms that mediate changes in the GH/IGF growth axis under different metabolic or nutritional states are not fully understood at present because the growth signaling system is a very complicated network and many factors are involved in this phenomenon process (Triantaphyllopoulos et al., 2020).

The liver is the central organ for lipid metabolism and detoxification (Meng et al., 2019). Here, the observations of histological structure of the liver in the research support of the above conclusion. There was an abnormal lipid storage and deposit accompanied by an alteration in lipid metabolism at a transcriptional level, and oxidative stress in fish fed with diets supplemented with the higher concentrations of the used AV powder. The increase in the number and diameter of hepatic lipid vacuoles in fish fed with the AV 2.5% and AV 5% powder supplemented diets was observed. The overload of lipids, mainly triacylglycerol (TG), in the cytoplasm of hepatocytes (> 60%) resulted in severe steatosis. This might be due to the presence of transformed constituents in AV (such as aloin) that may block hepatic metabolic enzymes (Rabe et al., 2005). Similar results have been observed in gilthead sea bream fish fed with 60% soybean oilas replacement (Bouraoui et al., 2011). Several cases of AV-induced toxic hepatitis in human patients have been reported in recent years. However, the involved mechanisms have not yet been described in the literature (Lee et al., 2014). Several studies have shown that AV is considered as anti-obesogenic, Nevertheless, the effects of some bioactive fitocompounds like phytoestrogens (i.e., genistein) also present in AV can have different effects on lipid metabolism in fish and in mammals depending on the tissue (Balbuena-Pecino et al., 2020). Even contradictory results are found in different mammalian models (Park et al., 2009; Grossini et al., 2018).

The disruption in lipid metabolism promotes the progression of liver damage. Regarding the "multiple hit process" during liver injuries, the first "hit" is the “accumulation of fat”, suggesting that the change in lipids occurs as a first step in hepatic damage (Yang et al., 2019). Lipid accumulation results from the balance between lipogenesis and lipolysis, and many genes are involved in this process (Meng et al., 2019). In this sense, *fas* is known to play crucial roles in lipogenesis by catalyzing the synthesis of saturated long chain fatty acids from acetyl-CoA, malonyl-CoA, and NADPH thus, can have adverse influence on fish liver function because it can induce lipid accumulation (Bou et al., 2014). In our study, (*fas*) expression was up-regulated in fish fed with the AV 5% supplemented diet, although not accompanied by *6gpdh* expression increase, the NADPH supplying enzyme. In addition, *lpl* gene expression was down-regulated in fish fed with AV 5%. These results suggest that dietary AV powder supplementation could enhance hepatic *de novo* lipogenesis while reducing lipolysis of plasma TG. These observations were in line with those of Rahoui et al (2018) showing that impaired lipolysis and a reduced *hsl* expression would promote the storage of excess lipids in rats. In other study, it has been reported that AV gel treatment inhibits the porcine pancreatic lipase activity *in-vitro* through its binding ability to both the free-enzyme and the enzyme-substrate complex (Taukoorah and Mahomoodally, 2016). In fish, several transcription factors play an intermediary role in lipid homeostasis, by controlling the gene transcription of the enzymes involved in lipogenesis and lipolytic pathways (Cruz-Garcia et al., 2011). Considering the transcriptional factors studied here, it has been described that *lxrα* is involved in TG breakdown in fish tissues (Cruz-Garcia et al., 2012), and its induction increases lipid efflux and decreases lipoprotein uptake, thus avoiding excessive lipid accumulation (Kidani et al., 2012). *pparα* stimulates the mobilization and the degradation of FAs by β-oxidation in tissues particularly in liver (Wei et al., 2017). In

the current study, *lxrα* gene was significantly down-regulated by the three-tested concentration of AV supplemented diet and *pparα* gene was significantly down-regulated by AV 2.5% powder supplemented diet. In parallel a down-regulation of gene expression of *hdadh* was showed, an enzyme involved in FA β-oxidation (Paredes et al., 2015) together with a decrease in *fabp11a* expression, a binding protein known to facilitate the entry of FAs into the cell for oxidation or storage. The mentioned results are in agreement with the work of Tseng-Crank et al (2013), who reported that UP780 (the combination of AV inner gel powder standardized containing 2%-4% aloesin) reduced FA β-oxidation by the inhibition of the *cpta1a*, *pparα* transcription factor and some FA transporters genes' expression. The second hit during liver injury was considered to be "oxidative stress" (Ramachandran and Jaeschke 2018). In healthy hepatic cells, an appropriate enzymatic and non-enzymatic antioxidant system has been described as a mechanism to eliminate excessive ROS. Among the most frequently, used biomarkers of oxidative stress are the enzymes SOD, CAT and GST. The inhibition of SOD activity may be due to the direct interaction between the enzyme and the aloin from AV extracts. Aloin is known to inhibit the action of the metalloprotease by interacting with zinc and/or calcium at the secondary site of the enzyme, which leads to a destabilization of its structure, which in turn enhances the production of O_2. radicals inside the cell and as a result of cell injury (Liang et al., 2021). Several studies have shown the prooxidant effect of this molecule from AV. It reduces (Fe^{3+}) to (Fe^{2+}) from Fenton reaction, which increases the generation of the hydroxyl radical (OH.) and (H_2O_2) (Nowak et al., 2021). The enhancement of CAT activity by AV treatment could be due to the presence of free radicals like H_2O_2, generated by the effects of aloin and its derivatives. PSHs plays a protective role against free radicals because of their sulfhydryl SH unit. In this study, the level of PSH proteins was reduced by the treatment of AV

at 2.5% level of inclusion. This could be due to the presence of excess of H_2O_2. Sutariya and Patel (2017) demonstrated that excessive H_2O_2 could block SH groups leading to the loss of SH units' reactivity, which ultimately induce toxicity (Smith et al., 2019). GSTs catalyze the conjugation of reduced glutathione (GSH) via the SH group, to electrophilic centers on endogenous compounds (Pavlidi et al., 2018). The GST activity induction in fish fed with AV 5% supplemented diet could play an important role in the protection against harmful aloin metabolites generated after the metabolization (Yan et al., 2016). Several studies have shown that the depletion of antioxidant enzymes leads to ROS accumulation and the development of liver disease (Kim et al., 2020). The reduction of SOD activity and the increase of TBARS levels in fish fed with diet supplemented higher concentrations of AV powder could be the consequence of the high generation of ROS like the hydroxyl radical generated by aloin. Being the specific target of ROS, macromolecules namely lipids could be easily oxidized to produce malonedialdehyde (MDA), a marker of lipid peroxidation and induce structural and functional alterations of cell bio-membranes (Yan et al., 2015). Support to this assumption is given by (Buenz, 2008) who showed that the treatment of Jurkat cells with aloin, dose-dependently reduced cell size, disrupt and blocked the cell cycle at the G2/M phase. Nevertheless, after 0.5% AV treatment the parameters related to hepatic oxidative status were not significantly affected.

Concerning signaling pathways, *akt* and *mtor* are molecules known, among others, to improve the efficiency of global protein synthesis and stimulate hepatic lipogenesis (Zhang et al., 2019). *mtorc1* is a serine/threonine kinase that plays a central role in many processes such as protein and lipid synthesis and, at the same time, it also suppress autophagy, therefore controlling the balance between cell anabolic and catabolic pathways (Saxton et al., 2017). Here, the incorporation of AV powder in the fish diet induced significantly a down-regulation

of *mtor*. It has been shown that mtorc1 promotes *de novo* lipid synthesis through the activation of sterol responsive element binding protein (*srebp1c*), a transcription factor that activates numerous genes involved in lipid biosynthesis (Pan et al., 2019). On the other hand, the reduction of *mtorc1* gene expression causes an impairment in oxidative capacity, and induces autophagy in tilapia (Han et al., 2020). Furthermore, the *mtor* pathway played an important role in the inflammatory response in fish (Tan et al., 2018). The inhibition of *mtor* signaling pathway increased proinflammatory cytokines such as *il-6, tnf-α,* inducing apoptosis in hybrid grouper (Tan et al., 2018). Therefore, the lower levels of *mtor* observed in our study could contribute to the impairment of oxidative status and hepatic injury in gilthead sea bream. However, the decrease in *mtor* could be in fact a tissue response to reduce fat accumulation, since several studies showed that rapamycin, the inhibitor of *mtorc1,* inhibits lipogenenic enzymes in trout (Dai et al., 2013), and also alleviates hepatic steatosis caused by fructose in zebrafish (Han et al., 2020). The protein kinase B (*akt*) activation has been shown to increase lipogenic genes expression. In fact, the inhibition of *akt* activation blocks the increase in mRNA expression of the *srebp1c* gene (Smith et al., 2008). Our findings indicate that hepatic *akt* gene expression was decreased by AV treatment reflecting presumably an adaptive cellular mechanism to also reduce liver steatosis. A recent transcriptional study showed that the suppression of *akt* driven Ribosomal protein (RPS) phosphorylation and *srebp1c/ fas* mediated lipogenes is alleviate hepatic steatosis (Zhang et al., 2019). Next, *erk1/2* are part of the family of Mitogen activated protein kinases (MAP kinases), proteins that play an important role in cell division, growth and proliferation (Chen et al., 2018; Mahali et al., 2014) and stimulate the phosphorylation of *srebp1c*. In the current study, an inhibition of the expression of *erk1/2* by the tested concentrations of AV was noted contributing to the regulation of lipogenesis. Alternatively,

such inhibition might be attributed, at least in part, to the active AV component aloin as previously reported in mouse (Zhong et al., 2019).

In summary, gilthead sea bream (Sparus aurata) fed with diet supplemented with higher concentrations of AV powder showed an induction of oxidative stress and lipid peroxidation, alteration of the expression of genes involved in lipid metabolism and cellular signaling pathways, and high hepatic lipid deposition as revealed by histological analysis.

The principal component analysis of the molecular, biochemical and histological responses of the fish (*Sparus aurata*) fed with the increasing concentrations of AV showed that the principal component 1 (PC1) is positively correlated with antioxidant markers (SOD and PSH) and expression levels of genes involved in lipid metabolism and regulation (*hsl, pparα, hadh, g6pdh, lxrα, fabp11, mtor*). This means that by adding the AV 2.5% supplemented diet, the animal responds through several biochemical and molecular pathways to combat this addition. The principal component 2 (PC2 axis) (24.68% of the total variance) is positively correlated with the biochemical variables (GST and MDA) and the lipogenic genes (*fas*) and (*igf-II*) and clearly separate the control group from the AV 5% group. This means that increasing AV concentrations in the diet of fish altered the biochemical and molecular parameters including the disruption of antioxidant status (PSH and SOD), extended lipid peroxidation. These alterations are responsible for an abnormal lipid metabolism in hepatocytes by reducing the expression of genes involved in lipolysis (*hsl, atgl*), those involved in the catabolism of FAs (*ppar, hadh*) in particular AV 2.5% and the increase in that of genes involved in lipogenesis such as *fas* by the AV5% then causing hepatic steatosis.

5. Conclusions

In summary, we have evaluated the effects of different concentrations of dietary AV supplementation on gilthead sea bream growth performance, antioxidant status, liver histology and lipid metabolism related genes' expression. We found that supplementation at 0.5% enhanced hepatic IGFs levels and expression without affecting the other studied parameters. However, AV at 2.5 % and 5% caused oxidative stress, and reduced the efficacy of liver FA catabolism, and cell survival pathways-related genes, while increased FA synthesis-related genes' expression, which is associated with hepatocellular excessive lipid accumulation and as a result, hepatic steatosis, and this might be due to the identified anthraquinones in AV powder.

Author contributions

The authors thank the participants who gave their time to the trial. Jamel JEBALI and Isabel NAVARRO designed, supervised the study and revise manuscript. Afef AMRI designed, performed experiments, analyzed data and co-wrote the paper. Zied BOURAOUI contributed to animal experiments, samples collection and analyzed data. Sara BALBUENA-PECINO and Encarni CAPILA contributed to molecular analyses and revise manuscript. Hamadi GUERBEJ carried out the rearing work. Karim HOSNI supervised the phytochemical analysis and revise manuscript. Tahar GHARRED and Zohra HAOUAS contributed to the histological and statistical analysis of the results. All authors analyzed the results and contributed to the manuscript writing, and approved the final version of the manuscript.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declaration of Interest Statement

The authors confirm that there are no conflicts of interest in this work.

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Ethical approval

All experimental procedures complied with the guidelines regarding the use of laboratory animals of the Bioethical Commission of Scientific Research of the Higher Institute of Biotechnology of Monastir- Tahar Haddad Street, (B.P 74), Monastir, 5000-Tunisia.

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FIGURE CAPTIONS

Figure. 1: Hepatic oxidative stress parameters of gilthead sea bream fishes fed with diets containing 0, 0.5%, 2.5% and 5% of dietary *Aloe vera* (AV) powder for eight weeks. (A) Catalase (CAT) and Glutathione-S-trensferase (GST), (B) Protein sulfhydryl (PSH) and Thiobarbituric acid reactive substance (TBARS) levels Data represent means ± SEM). (10 fish/treatment). Different letters indicate significant difference among between fish groups (one way ANOVA and Duncan's test, p< 0, 05).

Figure. 2: Growth factors (A) hepatic insulin-like growth factor level and (B) gene expression of insulin-like growth factor-I and II of gilthead sea bream fed the control and the experimental diets supplemented with 0.5%, 2.5% and 5% *Aloe vera* powder for 8 weeks. mRNA expression values were normalized to reference genes (*ef1a* and *rps18*) expressed as a ratio of the control groups. Data represent means ± SEM. Different letters indicate significant difference between fish groups (one way ANOVA and Duncan's test, p< 0, 05).

Figure. 3: Hepatic expression of genes involved in lipid metabolism in gilthead sea bream fed the control and the experimental diets supplemented with 0.5%, 2.5% and 5% *Aloe vera* powder for 8 weeks. (A): genes coding for enzymes related to lipogenesis [Fatty acid synthase (*fas*) and glucose-6-phosphate dehydrogenase (*g6pdh*)], (B): genes of enzymes involved in lipolysis [Lipoprotein lipase (*lpl*), hormone-sensitive lipase (*hsl*) and Adipose triglyceride lipase (*atgl*)], (C): gene of transcription factors (Peroxisome proliferator-activated receptor (*pparα*) and selected enzyme (Hydroxyacyl-Coenzyme A dehydrogenase (*hadh*) involved in β-oxidation pathway (D) genes of fatty acids transporters [cluster of differentiation 36 and fatty-acid-binding proteins (*fabp11*)] and (E): gene of transcription factor involved in cholesterol and fatty acid homeostasis [Liver X receptor alpha (*lxrα*)]. mRNA expression values were normalized to

reference genes (*ef1a* and *rps18*) expressed as a ratio of the control groups. Data represent means ± SEM. (6 fish/treatment). Different letters indicate significant difference between fish groups (one way ANOVA and Duncan's test, p< 0, 05).

Figure. 4: Gene Expression of protein kinases involved in cellular signaling pathways. [RAC-alpha serine/threonine-protein kinase of gilthead sea bream fed the control and the experimental diets supplemented with 0.5%, 2.5% and 5% *Aloe vera* powder for 8 weeks. (*akt1*), mammalian target of rapamycin (*mtor*) and extracellular-signal-regulated kinase (*erk1/2*). mRNA expression values were normalized to housekeeping genes (*ef1a* and *rps18*) expressed as a ratio of the control groups. Data represent means ± SEM (6 fish/treatment). Different letters indicate significant difference between fish groups (one way ANOVA and Duncan's test, p< 0, 05).

Figure. 5: Hematoxylin-eosin staining of liver tissues of gilthead sea bream fed with control (A) and experimental diets supplemented with (B) a): 0.5% *Aloe vera* powder, (C): 2.5% *Aloe vera* powder (D): 5% *Aloe vera* powder for 60 days. HP hepatopancreas, H hepatocytes, V vacuolization, the magnification was × 40 N = 9 fish/treatment and 3 sections per fish were analyzed.

Figure. 6: Principal component analysis (PCA) performed on the biochemical and molecular variables of gilthead sea bream fed the control and the experimental diets supplemented with 0.5%, 2.5% and 5% *Aloe vera* powder for 8 weeks.