Advances in Bioscience and Biotechnology, 2016, 7, 55-62
Published Online February 2016 in SciRes. http://www.scirp.org/journal/abb
http://dx.doi.org/10.4236/abb.2016.72007

Fish Nutrition Additives in SHK-1 Cells: Protective Effects of Silymarin

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Received 9 January 2016; accepted 15 February 2016; published 18 February 2016

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Abstract

In nutrition for productive species, additives play an important role in boosting physiological processes. Only in recent years studies include models of the effects on fish cells of these additives. We observed effects of silymarin, a compound highly utilized in aquaculture. The cell line SHK-1 was used derived from the upper liver of the Atlantic salmon as a biological model. Samples were exposed to silymarin in incrementing time and concentrations, to evaluate by MTT and number of cells, the effects on cell viability. Also, oxidative stress models were used to find the protector effects of silymarin against these agents. Our data indicate that a dose of 100 ppm of silymarin is sufficient to stimulate cellular proliferation. Cultures were exposed to high glucose (15 mM) or H_2O_2 (0.1 mM) in presence of or absence of silymarin at 100 ppm. We observed that the toxic effects of both compounds were blocked by the presence of silymarin. Our results indicate that it is important to evaluate additive effects at a cellular level. Also, silymarin does have proliferative effects, and protect against cellular injury in our models. Our study helps to generate more rational applications of additives in the industry and presents new challenges in order to better manipulate the model in the laboratory, allowing us to obtain new evidence regarding the microalgae's biology through *in vitro* studies.

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How to cite this paper: Sanchez, R., Olivares, P., Carmona, E., Astuya, A., Herrera, H. and Parodi, J. (2016) Fish Nutrition Additives in SHK-1 Cells: Protective Effects of Silymarin. *Advances in Bioscience and Biotechnology*, **7**, 55-62.
http://dx.doi.org/10.4236/abb.2016.72007

Keywords

Fish, Nutrition, Silymarin, Cell Cultures

1. Introduction

Cellular cultures are important for fish research and in vertebrates, albeit in the exploration of additives or their interaction with organs and the appearance of secondary effects. Cell cultures also provide unlimited biological material for the diagnostic of alterations produced by additives [1] [2]. Cell lines are study models for molecular effects' con cell function [3]. In vertebrates, especially mammals, they have been amply utilized for biotechnology development, such as the CHO cells [4]. Although it is novel in aquaculture, the passage from information at a cellular level to complex models common in mammals [3]. The most developed compound search is in frog (*X. laevis*) oocytes, where endogenous receptors are used to understand the pharmacology of nicotine receptors [5]. For this same reason, understanding additive effects at a compound cellular level in fish models is a good option to improve the comprehension of the physiology of them, and avoid toxicity through chronic applications [6] [7]. Silymarin is a complex flavonoligan of *Silybum marianum* (L.), commonly called thistle, and that comprehends a large number of flavonoligans including Silybin (silybin A and B) isosilybin A and B, silychristin A and B, silydianin and other phenolic compounds [8]. It is amply used in the treatment of hepatic diseases in vertebrates [9] which reduces toxic effect on liver [10] and has effect over hepatic system in fish [11]. Literature suggests that the antioxidant properties of silymarin contribute to its pharmacological properties [12]. But this is not its only exclusive mechanism [13] [14]. Apart from antioxidant properties, it has anti-lipid peroxidative, anti-fibrotic, anti-inflammatory, membrane stabilizing, immunomodulation, as well as anti-tumoral properties, and shows anti-arteriosclerotic and anti-diabetic activity [15] [16]. In fish, the use of silymarin as an antioxidant is not widely studied [17], and it is used in diets as an additive that boosts development and growth [18]. Its use in diets is diverse; it can be used in the farming of freshwater fish [19], or in the fattening of saltwater fish [18]. It has been used as an immunomodulator [15] or a protectant against agents such as curcumin [17], and can regulate function in fish glial cells [20]. Its action mechanism in fish is unknown, and is identified as an additive that is absorbed as a flavonoid through the intestinal wall. Once in the bloodstream, it plays a role in improving cellular nutritional conditions. This could be because of undescribed antioxidant properties, or due to being a metal-chelating agent, improving cell function and development in fishes [15] [17].

In this study, SHK-1 derived cells from the head and kidney of Atlantic salmon were treated in increasing time and concentrations of silymarin. Then, these samples were analyzed by MTT for the number of cells in response to proliferation and their viability. Also, models of oxidative stress were used to measure the protective capacity of silymarin against cells exposed to high levels of glucose.

2. Methods

2.1. Cellular Cultures and Cytotoxic Studies

Cell lines derived from leucocytes from Salmo salar SHK-1 (ECACC N˚97111106), were maintained in an incubator at 17˚C. Cells were cultivated in a Leibowitz L-15 medium and supplemented with 10% fetal bovine serum, glutamine 1%, penicillin/streptomycin 1% and 2-mercaptoetanol 72 µl/ml. The cellular expansion procedures were carried out in a biosafety cabinet and then are seed in plates of 24 wells for parallel experiment. Cultures were exposed to temporal curves (one to seven days) and concentrations (0.1 to 1000 ppm) of generic silymarin to evaluate its toxicity, staining and morphology were analyzed to explore the reaction to the additive. Modifying [21], the effect was added of high glucose 30 mM at for 24 hours to generate free radicals, and for seven days to generate an oxidative state. Also, 100 µM de H_2O_2 was used for 24 hours and 7 days to observe the direct effect of free radicals and the antioxidant action of the additives.

2.2. Staining

Cells grown in culture plates were exposed to temporal curves and concentrations of the additives. Cells were then washed of the culture medium and incubated with eosin 0.3%, glacial acetic acid and distilled water for one

minute. Then, then solution was removed and a phosphate buffer was added. One hundred cells were counted randomly, and those that presented a reddish-orange tone were considered dead, and this percentage was recorded. The methodology was based on [22] and modified.

2.3. Cellular Proliferation

Cells were washed with PBS and removed with a trypsin solution 1%, later they were placed in complete culture medium to inhibit the trypsin. Samples were collected in sterile 15 ml tubes and were centrifuged at 1200 rpm for 10 minutes. Cells in the bottom were suspended in 1 ml of base medium and a sample was taken to be recounted in a haemocytometer diluted 1/100. With this value, samples were seeded in at a density of 100,000 cells per ml, in culture with 1.6 mm diameter, with 250 ul of medium. This culture was exposed for 24 hours to the additives and the number of cells was recounted by area. The density of cells was recorded as an indicator of the change in cellular proliferation or growth rate, modified from [23].

2.4. May-Grunwald and Giemsa Staining

Cells grown in culture plates were exposed to temporal curves and concentration of additives were washed of culture medium and incubated with a PanOptic kit for May-Grunwald and Giemsa staining (Quimica Clinica Aplicada, Spain). Samples were left for 5 seconds in reactive 1, 10 seconds in May-Grunwald reactive and 10 seconds in Giemsa reactive. The plates were washed with ultrapure water and left to be microphotographed with a NIKON Labophot 2 optic microscope and a 519CU 5.OM CMOS camera, modified from [24].

2.5. Statistical Analysis

Unless specified, all results including image analysis are presented using the average ± SEM, we used prism 5 software for the analysis. Statistical comparisons were carried out using ANOVA two way, indicated in the figure legend and Bonferroni test, are used after the analysis (post-test). The sample are exposed to normality test, Shapiro-Wilk, for see how for the distribution is from Gaussian. A probability p less than 0.05 is considered statistically significant.

3. Results

3.1. Silymarin Promotes Cell Proliferation in a Concentration-Response Manner

The cells are seeded and the beginning of the experitmen observed at time = 0 (initial condtion) that the number of cells per area upon adding 150 ppm of silymarin. **Figure 1(a)** shows an example image of the control condition and the cultures treated with 150 ppm silymarin from time = 0 and at 5 days of exposure, showing an increased number of cells per area in the exposed sample. **Figure 1(b)** represents a quantification of cell numbers per area at different times, and an increase in cell number can be observed in the treated sample. **Figure 1(c)** shows the quantified effect of 150 ppm of silymarin. **Figure 1(d)** shows that there was no deleterious effect on the culture upon being acutely exposed. These data suggest that a dose of 150 ppm does not acutely affect the culture, but it increases chronic cell proliferation.

3.2. Concentration Dependent Effect and Toxic Model

We evaluated the concentration-dependent effect for silymarin. For this, we made proliferation curves with different doses of silymarin (0.1 - 1000 ppm) and we observed the number of cells at the end of the proliferative curve. We observed at 5 days had the maximum proliferation effect and we used the number of the cells at these time for see the effect of the different concentration of the silymarin in the proliferation. In **Figure 2(a)**, a sigmoidal curve is presented of the effect, where 1000 ppm of silymarin is the concentration to generate the median cellular proliferation effect. In mammals, it is described that an increase in metabolism generates oxidative stress conditions, and a manner to induce these states is by using glucose. We did not know what the effect of glucose was on the SHK-1 model, being a culture that is developed at a lower temperature. For this purpose we measured the viability of the culture using MTT at different concentrations of glucose on the external medium for 24 hours. **Figure 2(b)** shows the development of a curve, indicating that concentrations of 30 mM of glucose generates a significant decrease in the viability of the culture, and can bused as a cellular injury model for oxidative stress.

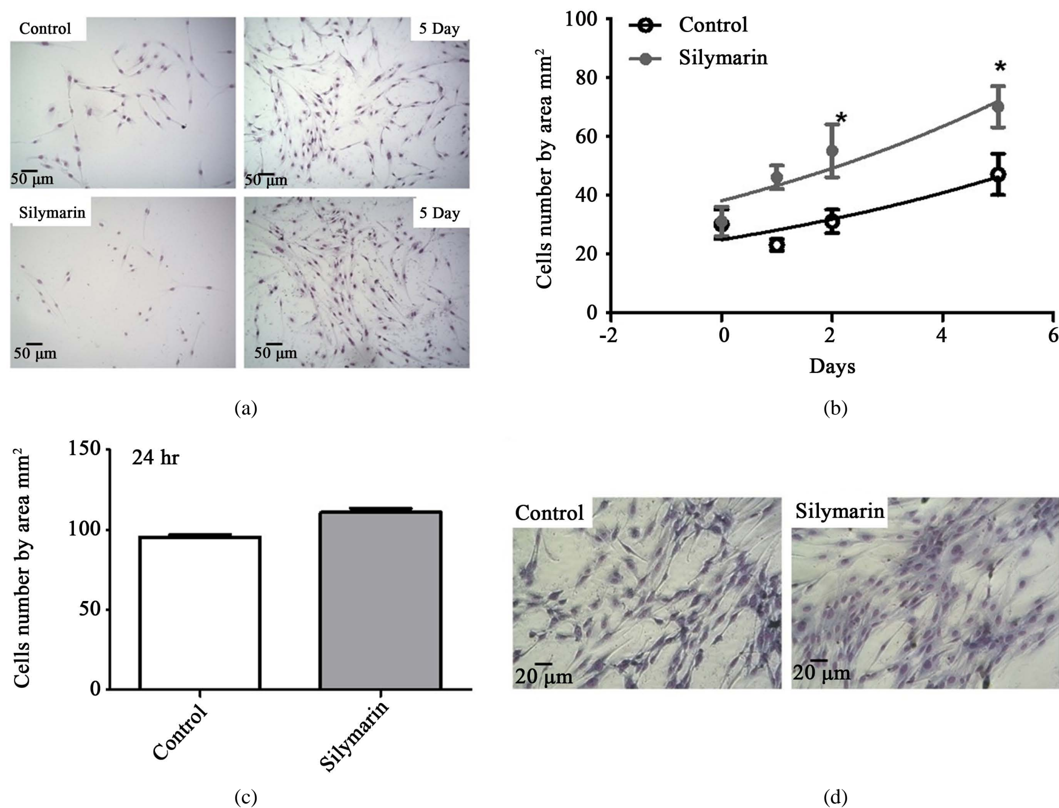

Figure 1. Effect on cellular proliferation. In (a), example of microphotographs in control, and with silymarin 150 ppm at times 0 and 5 days; (b) quantification of the number of cells after 5 days of culture in absence or presence of silymarin; (c) shows the quantification of the number of cells of the culture exposed to 150 ppm of silymarin after 24 hours; (d) is representative of the quantification at 24 hours of treatment with silymarin 150 ppm. The microphotographs are representative of the 5 independent observations. Each bar or point represents (mean ± SEM), la measurements of at least 5 independent culture and experiments. The asterisk indicates $p < 0.05$ (ANOVA).

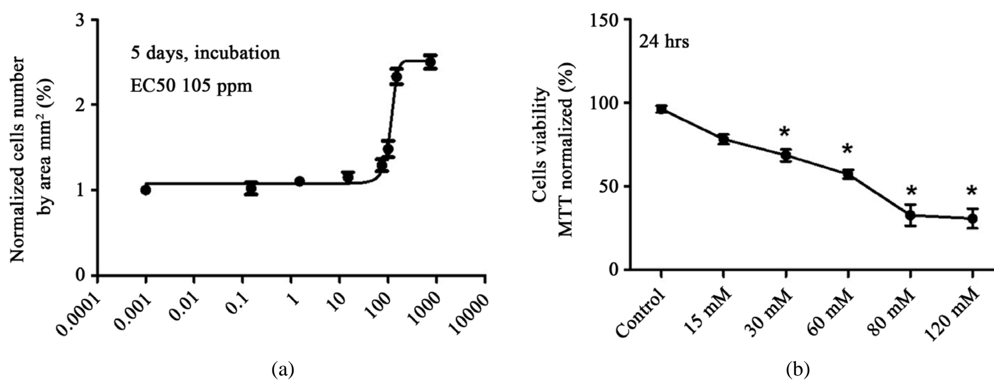

Figure 2. Doses concentration response. In (a), doses concentration curve, proliferation at 5 days of silymarin 0.1 to 1000 ppm. An EC of 105 ppm is observed for proliferation; (b) shows cellular viability of cell cultures exposed to increasing concentrations of glucose, 15 mM to 120 mM, for doses of toxicity at 24 hours of treatment. Each point represents (mean ± SEM), the measurement of at least 3 independent culture and experiments. The asterisk indicates $p < 0.05$ (ANOVA).

3.3. Silymarin Reduces the Effects of High Glucose

Observing that glucose at 30 mM alters the viability of the cultures, we tested its effect when it was maintained in the presence of 100 ppm silymarin. Figure 3(a) shows samples that were exposed for seven days to high glucose, in the absence and presence of silymarin. Figure 3(b) shows the quantification of the number of cells ex-

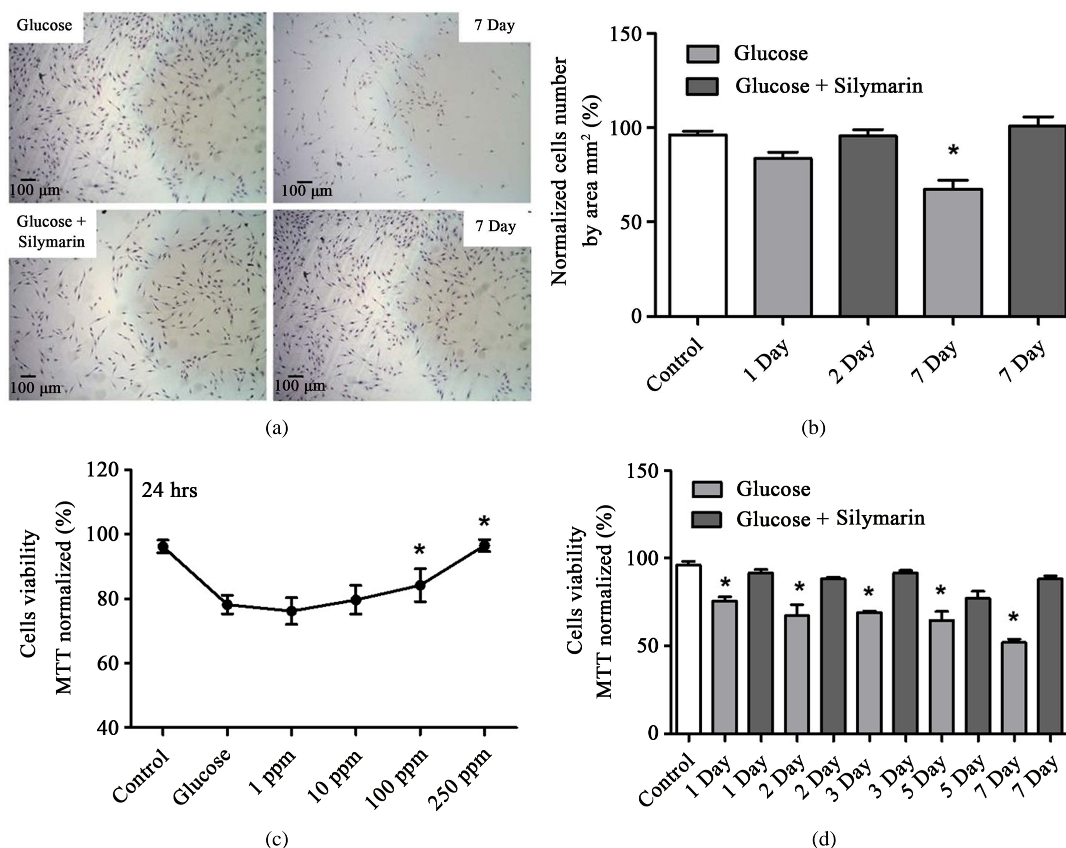

Figure 3. Cellular protect effect. In (a), example of microphotography H_2O_2 0.1 mM in the presence or absence of silymarin 100 ppm, 7 days of incubation; (b) demonstrates the quantification of the cells after 7 days of culture in H_2O_2 0.1 mM in the absence or presence of silymarin 100 ppm; (c) gives the effect of H_2O_2 0.1 mM after 24 hours on cell viability in the absence or presence of increasing concentrations of silymarin. Microphotography is representative of 6 independent observations made in 6 different cultures. Each bar or point represents (mean ± SEM), the measurement of at least 6 independent culture and experiments. The asterisk indicates $p < 0.05$ (ANOVA).

posed to glucose in absence of precense of silymarin. We observed a reduction on the toxic glucose effect, when exposed to 100 ppm of silymarin. With the objective of determining if the effect is concentration dependent, cultures exposed to 30 mM of glucose for 24 hours, were incubated in incrementing concentrations of silymarin (1 to 250 ppm). As is shown in **Figure 3(c)**, we observed that very low concentrations of silymarin did not revert the deleterious effects of the glucose, and that values over 100 ppm protect the culture. Upon using the concentration in a chronic form, and evaluating viability by MTT, as is shown in **Figure 3(d)**, we observed that 100 ppm is sufficient to significantly reduce the effect of 30 mM of glucose on the cells. These data suggest that silymarin is capable of reducing an environment of cellular stress generated by high glucose.

3.4. Silymarin Reduces the Effects of Hydrogen Peroxide

The metabolic effect, associated to a culture with high glucose, is possible to associate to generation of free radicals that alter the REDOX of the cell. To confirm that silymarin can participate in reduces this effect, we used H_2O_2 directly on the culture, to generate an intense toxic effect, suggesting an oxidative stress state. **Figure 4(a)** shows images of the cultures exposed to H_2O_2 0.1 mM in the absence and presence of silymarin 100 ppm. **Figure 4(b)** shows a quantification of the chronic effect of the H_2O_2 on the culture in the absence and presence of silymarin, observing that after 7 days of co-incubation, the number of cells increased with respect to the culture exposed to H_2O_2 in a solitary form, although still less than the control. Upon evaluating the viability of the culture by MTT in acute applications, we observed that silymarin reduces the effects of H_2O_2 in a concentration dependent form (**Figure 4(c)**). Concentrations less than 100 ppm did not significantly reduce the mortality of the

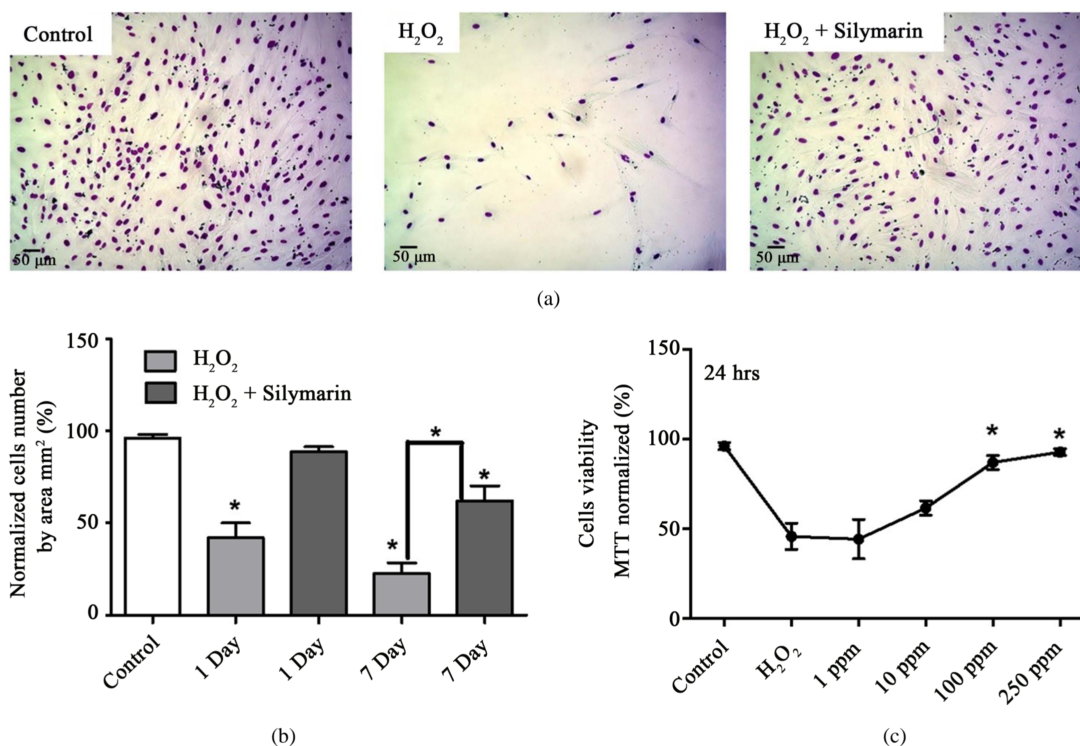

Figure 4. Cellular protect effect. In (a) example of microphotography H_2O_2 0.1 mM in the presence or absence of silymarin 100 ppm, 7 days of incubation; (b) demonstrates the quantification of the cells after 7 days of culture in H_2O_2 0.1 mM in the absence or presence of silymarin 100 ppm; (c) gives the effect of H_2O_2 0.1 mM after 24 hours on cell viability in the absence or presence of increasing concentrations of silymarin. Microphotography is representative of 6 independent observations made in 6 different cultures. Each bar or point represents (mean ± SEM), the measurement of at least 6 independent culture and experiments. The asterisk indicates $p < 0.05$ (ANOVA).

culture. These date suggest that silymarin acts as protect, reducing the effect of H_2O_2 on the cultures in concentrations superior to 100 ppm.

4. Discussion

The use of cells for the study of additives is a widely used tool in mammalian studies [3]; recently, this type of testing has started to be used in fish, as a way to evaluate procedures [6] and obtain functional information about additives [15]. Our results were obtained using SHK-1 cells exposed to silymarin, an additive used in the salmon industry.

We found that silymarin did not alter cellular viability, and promoted proliferation in this cell line (**Figure 1**), as well as proliferation in a dose response manner with a value of EC50 of 105 ppm (**Figure 2(a)**). Silymarin is described as being a good antioxidant in other cellular model [12] and can complete this function in fish [17]. We exposed the effects of silymarin on cultures exposed to high glucose, for made simulation of high oxidative stress (**Figure 2(b)**), which has been previous described in other cell model [25]. We found that the effects of glucose were reduced when the culture was exposed to 100 ppm silymarin (**Figure 3(b)**), and this effect in dependent concentration shows that values of 100 ppm of silymarin protect the culture.

Our objective was to determine the direct effect of free radicals on SHK-1 cultures, and if silymarin can reduce their toxicity. So, we exposed the cultures to 0.1 nM of H_2O_2 in the presence and absence of 100 ppm silymarin (**Figure 4(b)**). We observed that the silymarin reduced the mortality of the culture, suggesting a protective effect on oxidative stress conditions. We found that this effect is also concentration dependent (**Figure 4(c)**), as observed with glucose; values less than 100 ppm did not reduce the effect of H_2O_2, increasing mortality in these conditions.

Our findings indicate that concentrations of 100 ppm of silymarin are sufficient to promote cellular division in SHK-1 cells and significantly reduce the toxic effects in cellular injury model. This suggests that in these condi-

tions, silymarin is an additive which has functions as a cell protector. These results are applicable to the formulation of fish diets, and further studies should focus on the bioavailability of silymarin and the effect it could have on aquaculture.

Acknowledgements

Financed by the Convenio de Asistencia Técnicas UCT 278-2450 Tonalli 2013. Jorge Parodi received the grant MECESUP UCT 0804. We thank the business SalmoFood-VitaPro, for donating the additives for testing. Rodrigo Sánchez, postgraduate student at the UCT received financing as part of his thesis from SalmoFood-VitaPro.

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