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5. Evaluation of anti-inflammatory activity of food compounds using zebrafish

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Abstract. The principal aim of this work was to optimize and apply a zebrafish experimental model for the screening of anti-inflammatory substances present in the Mediterranean diet. The zebrafish is an organism widely used in various fields of experimental biology. The inflammation is easily inducible, reproducible and visualized in their early stages of development. Specifically, the migration of neutrophils to the injured caudal fin, one of the first steps of the inflammatory response, is quantitatively measured by image analysis. The anti-inflammatory effect of natural compounds can be evaluated as a decrease of migration. Adverse effects triggered by inflammation are mainly mediated by reactive oxygen species. The anti-oxidant activity of compounds was evaluated in zebrafish embryo measuring their protective effect against tert-butyl hydroperoxide toxicity. Several phenolic compounds have been assayed. Our results showed that the compounds with the greatest decrease on neutrophil migration were chlorogenic acid and cyanidin. The activity of these two polyphenols

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was quite similar to that observed with anti-inflammatory drugs (indomethacin, piroxicam) and NADPH oxidase inhibitor compounds (dibenzoidolium, apocynin). The anti-inflammatory and the anti-oxidant activity of the assayed polyphenols did not show a clear correlation.

Introduction

Nutrition is one of the main determinants of health. Multiple studies show that a balanced diet contributes to the prevention of numerous diseases. Specifically, the so-called "Mediterranean diet" has been associated with benefits in the prevention of many of the most prevalent chronic diseases in Western societies; it is known that the Mediterranean diet provides important dietary components such as carotenoids and phenolic compounds, which can contribute to reduce the risk of developing different pathologies [1].

Many of these chronic diseases (cardiovascular, metabolic, neurodegenerative) have an important component of inflammatory type in their etiology and pathological mechanism. Inflammation arises as a response of the body's immune system to cellular and tissue damage, and involves a cellular component and a chemical oxidative component. The cellular component involves the migration of leukocytes, mainly neutrophils, from the blood vessels to the injured tissue, attracted by several molecular factors liberated by the damaged cells and by the tissue resident macrophages. Once the leukocytes reach the site of the injury, a series of processes are triggered that include the formation of free radicals and the degradation of phospholipids to arachidonic acid (AA) by the action of phospholipases; arachidonic acid is metabolized by the cyclooxygenase or lipoxygenase pathway, thus producing prostaglandins, leukotrienes and thromboxane, inflammatory mediators necessary in the inflammation process [2]. Adverse effects of inflammation are mainly mediated by cytokines, proteases and oxygen species released by immune cells [3]. Therefore, the migration and activation of immune cells must be finely controlled because an excess of activity can cause additional tissue damage.

Neutrophils play a main role in the first steps of inflammation. They are the leading cells in host defense responses. The early recruitment is guided by tissue damage-associated molecular patterns (DAMPs) recognized by specific Toll-like receptors. One of these signal molecules is hydrogen peroxide generated by specific NADPH oxidases of injured epithelial cells. For more sustained recruitment, additional long-range

signals are activated, mainly chemokines of the CXCL8 family. These chemokines are produced both by immune cells (neutrophils, macrophages, T-cells) and tissue endothelial and epithelial cells. Lipids derived from arachidonic acid are also strong inducers of neutrophil chemotaxis.

Neutrophils are difficult cells to manipulate, therefore a series of techniques are used to understand their biology. Among the models used to investigate the biology of neutrophils are purified human neutrophils, neutrophil cell lines, murine models and zebrafish. In the zebrafish larvae is possible to study the migration of neutrophils in response to a tissue injury in the whole organism environment [4].

Like mammals, teleost fishes possess several types of granulocytes and a separate lineage of macrophages. The most abundant granulocyte in the zebrafish is the neutrophil, which is characterized by having a segmented multi-lobed nucleus (2-3 lobes). This cell is similar to the human neutrophil, which also has a nucleus of multiple lobes and a heterophilic cytoplasm. As the neutrophil matures, so do its granules, expressing and accumulating several enzymes necessary for its function including myeloperoxidase. Polymorphonuclear neutrophils are the leukocyte dominant in zebrafish larvae at 4 days post fertilization (dpf), and in adult mammals [5].

Diet plays an important role in maintaining an optimal immune response, so that deficient intake can have negative consequences on the immune status and susceptibility of the organism to a wide variety of pathologies. The mechanisms implicated could be mediated by specific regulatory effects on the cellular response, or by an unspecific "antioxidant" action [6]. The present study is focused on investigating the application of a zebrafish larvae model for the screening of natural components present in the Mediterranean diet with possible anti-inflammatory activity. The zebrafish is an organism widely used in various fields of experimental biology. This animal model has multiple advantages such as high fecundity, small size, low maintenance cost, high throughput, rapid extra uterine development and the optical transparency of embryos and larvae. In addition, the exposure needs small amounts dissolved in the surrounding medium and compounds are absorbed through the gastrointestinal tract or through the skin, which allows a rapid evaluation of pharmacological or toxicological activity in vivo [7-10].

Two types of assays were developed and applied. In the first assay, the capacity of the substance to prevent the migration on neutrophils was measured. In the second assay, the in vivo "antioxidant" activity was evaluated measuring the protective effects against an oxidative stressor.

1. Material and methods

Obtaining zebrafish larvae

Adult wild type zebrafish were kept under standardized conditions. Eggs were collected cleaned and selected. Fertilized embryos were treated with standardized water, according to ISO 7346-1 and 7346-2 (2mM $CaCl_2.2H_2O$, 0.5mM $MgSO_4.7H_2O$, 0.75mM $NaHCO_3$, 0.07mM KCl). The fish were kept under controlled environmental conditions of 28ºC.

Solutions

Natural compounds and anti-inflammatory drugs (Sigma Aldrich) were dissolved in dimethyl sulfoxide (DMSO). From the stock solution, serial dilutions were made in Danieau's 0.3X medium (17.4 mM NaCl, 0.23 mM KCl, 0.12 mM $MgSO_4.7H_2O$, 0.18 mM $Ca(NO_3)_2$, 1.5 mM HEPES, pH 6.5) that do not contain more than 1% (v / v) of DMSO if used in larvae or 0.1% if used in embryo experiments.

Neutrophil migration assay

The neutrophil migration assay is based on the model described by Cordero-Maldonado *et al.* (2013) [11]. Zebrafish larvae of 4 dpf previously depigmented with 0.2 mM N-phenylthiourea (PTU) were used. Inflammation was induced by means of an injury in the tail fin and potentiated with the addition of lipopolysaccharide (LPS) 10µg / mL to the medium. Exposure to compounds was carried out for eight hours. First a pre-exposure of one hour was carried out, before tail fin cutting. Subsequently, larvae were exposed to the compound and LPS during 7 hours. Larvae were anesthetized and stained with Leucognost pox kit (VWR) following the instructions of the manufacturer. Neutrophils were stained since all the mature cells of the neutrophilic line are positive for myeloperoxidase activity. The tests were carried out in triplicate with larvae coming from different spawn with a whole minimum of 20-25 larvae per compound. Each test was compared with its own negative control to correct the model variability.

Stained cells were observed in the area of the lesion. The area covered by the stained cells in a selected region of the tail was used as the quantitative measurement of migration. A Nikon eclipse TS100 microscope was used to capture the images with a 10X magnification. The analysis of images was done with the program ImageJ. It consisted of the following

phases: first a single image was obtained from three images captured with different focus planes. Then the contrast of the image was increased applying an exponential transformation. A threshold was applied to eliminate the background and saturate the selected signal. Finally, the integrated intensity in a standardized region of the zebrafish tail was measured as shown in Figure 1. Since the signal was saturated, the intensity measured was proportional to the stained area.

Antioxidant activity assay

The protective capacity against oxidative stress in the whole organism was evaluated in zebrafish embryos. Embryos were exposed from 2 to 26 hours post fertilization (hpf) to the assayed compound or to solvent control medium. Afterwards, the medium was changed and embryos were exposed to several concentrations of tert-butyl hydroperoxide (tBOOH) (26-50 hpf) to induce oxidative stress. At 50 hpf the medium was changed to standard Danieau's 0.3X medium (Fig. 2). At 50 and 72 hpf the lethality and the presence of malformations was assessed and quantified following the procedure previously described by Teixidó et al (2013) [12]. The percentage of lethality and of dysmorphogenesis was calculated per compound at every tested concentration, and the concentration-response curves for these effects were plotted. From these curves the concentration of tBOOH which produced mortality to 50% of the embryos (lethal concentration 50, LC50) and the concentration at which 50% of the embryos presented at least one dysmorphogenic feature (effective concentration 50 for dysmorphogenesis, EC50) were calculated. The protective activity of the compounds was evaluated measuring the shift to the right of the lethality-tBOOH concentration and malformation-tBOOH concentration curves relative to the solvent control. The tests were carried out in triplicate with embryos coming from different spawn with a whole minimum of 25-30 embryos per compound and tBOOH concentration.

Statistical analysis

The results were expressed as the mean ± standard error of the mean (SEM). They were analyzed with the Graph Pad 7.02 Software Inc. program with a two-way ANOVA variance analysis and Bonferroni multiple comparison test. The results are shown as a percentage, where the controls correspond to 100% of neutrophil migration. In all cases, statistically significant differences are considered at three levels of significance, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

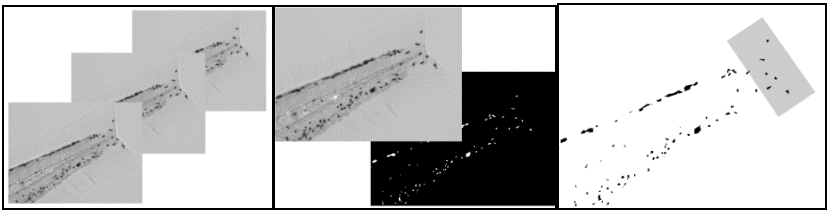

Figure 1. A) Images of zebrafish larvae showing cells with peroxidase positive staining captured with three different focus. B) Image before and after threshold application using ImageJ. C) Intensity integrated in a standardized area close to the fin tail.

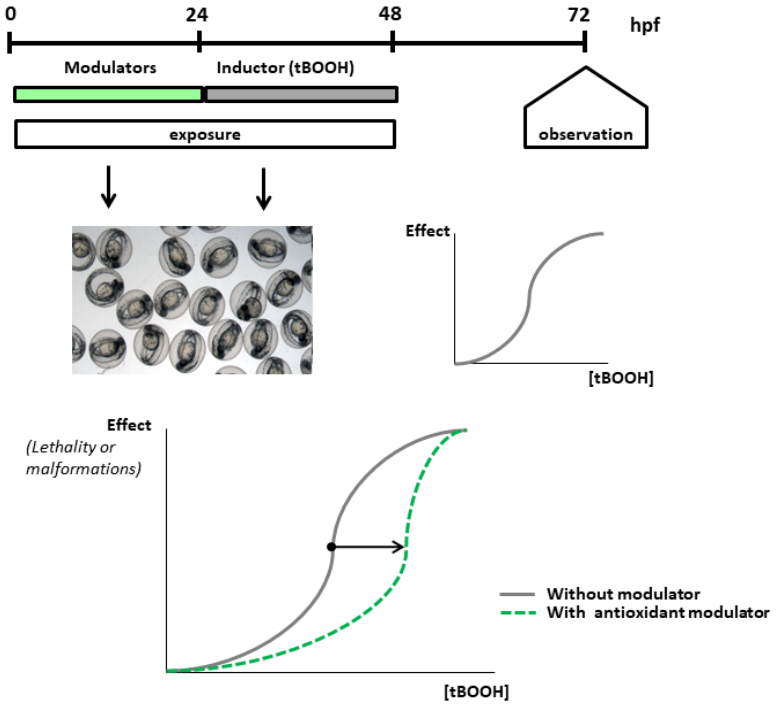

Figure 2. Experimental design of the antioxidant activity assay. Exposure to an antioxidant modulator protects from the toxicity induced by tBOOH. Shifts to the right of the effect (lethality or malformation index) *vs* concentration curve of tBOOH are observed.

Ethical statement

All procedures with zebrafish, larvae and embryos have been authorized by the Ethical Committee of Animal Experimentation of the University of Barcelona (protocol 7971 of the Department of Agriculture and Fishing of the Generalitat de Catalunya).

2. Results and discussion

Development and assessment of the methods

The capacity of the method to identify substances with anti-inflammatory activity was tested with some well-known positive controls, such as indomethacin and piroxicam, some non-anti-inflammatory products, such as doxepin and amantadine, and with inhibitors of NADPH oxidases such as dibenzoidolium (DPI) and apocynin (Fig. 3).

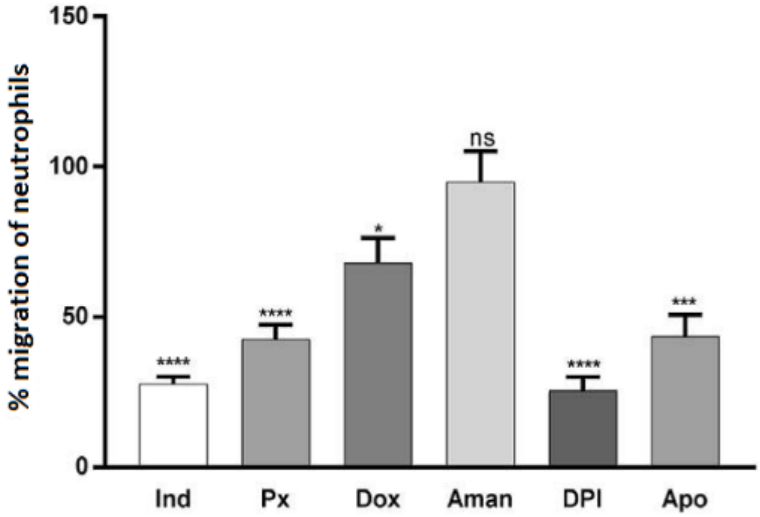

Figure 3. Migration of neutrophils, expressed in percentage of respective control (mean ± SEM). Larvae exposed to different anti-inflammatory substances such as indomethacin 100μM (Ind), Piroxicam 5μM (Px), non-anti-inflammatory compounds such as doxepin 5 μM (Dox), amantadine 10 μM (Aman) and inhibitors of NADPH oxidases such as dibenzoidolium 100 μM (DPI) and Apocynin 100 μM (Apo).

Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit both isoforms of the enzyme cyclooxygenase (COX). The COX-1 isoform is constantly expressed in most tissues, while COX-2 can be induced by various stimuli such as cytokines, prostaglandins and bacterial products such as lipopolysaccharide that contribute to the development of edema, fever and redness [13]. Our results of neutrophil migration in zebrafish larvae exposed to indomethacin and piroxicam show respectively 28% and 42% migration of neutrophils relative to the control.

Amantadine is an anti-viral drug generally used to treat and prevent influenza type A and without known anti-inflammatory activity. Treated larvae do not present significant differences with their control as the migration of neutrophils in this case represented 95%. The result is expected, since not being anti-inflammatory they have no effect on neutrophil migration. However, doxepin, which is an antidepressant drug, produced a moderate but significant inhibition of migration. Some studies suggest that doxepin has anti-inflammatory effects and is effective to treat atopic dermatitis [14].

The enzymes NADPH oxidases are dedicated exclusively to the production of reactive oxygen species (ROS). During phagocytosis, phagocytes activate NADPH oxidases to reduce molecular oxygen to superoxide anion, a precursor of microbicide reactive oxygen species (ROS). Previous studies suggest that after injury, cells in the wound margin of zebrafish larvae rapidly produce hydrogen peroxide (H_2O_2) that serves as an early paracrine signal to leukocytes [15, 16]. The test with DPI showed a significant inhibition of migration to 25% of controls. Apocynin, a phenolic compound isolated from the medicinal plant Picrorhiza kurroa, was also used, which also showed significant inhibition in neutrophil migration. Apocynin has been widely used to block the activity of NADPH oxidase in vitro [17, 18]. In our case, apocynin showed a 43% migration compared to controls.

These results suggest that the method could be applied for the identification of substances with potential anti-inflammatory activity. Significant inhibition of neutrophils migration was observed with substances acting at different molecular target (COX and NADPH oxidases) related with different chemotactic stimulus.

In order to validate the antioxidant activity assay, different compounds with well determined antioxidant activity were tested. A pre-exposure to N-acetylcysteine, Nω-Nitro L-arginine methyl ester hydrochloride (L-NAME), vitamin E, lipoic acid and quercetin resulted in significant shifts to the right hand of the lethality and malformation curves (results not shown).

Migration of neutrophils in zebrafish larvae exposed to food compounds

Tomato is among the most representative foods of the Mediterranean diet. Tomato derivatives, such as "sofrito" (fried tomato sauce, with onion, garlic, olive oil and some other vegetables), is characterized by containing a high variety of polyphenolic compounds [19]. We have assayed the activity of some of the major components, such as naringenin, oleuropein, rutin, cyanidin, chlorogenic acid and apigenin. The results showed that cyanidin and chlorogenic acid produce the greatest reduction of neutrophil migration (Fig. 4).

The putative protective effects of polyphenols on human health have been classically attributed to their antioxidant activity. Polyphenols can react directly with some reactive species. However it is now generally accepted that the direct scavenging of reactive radicals is not the main mechanism of action. A plethora of specific mechanisms have been described. Among

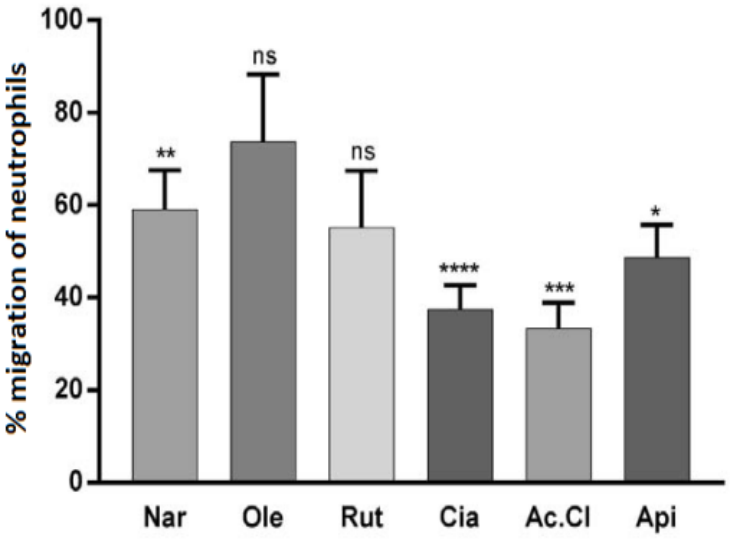

Figure 4. Neutrophil migration in larvae exposed to some polyphenols present in "sofrito". Naringenin 20μM (Nar), Oleuropein 150 μM (Ole), Rutin 20 μM (Ru), Cyanidin 20 μM (Cia), Chlorogenic acid 20 μM (Ac.Cl) and Apigenin 20 μM (Api). Data of each treated group is compared with its respective control and the percentage of migration is represented.

these mechanisms, several are directly related to potential anti-inflammatory effects. Inhibition of inflammasome activation and NF-kB inflammatory pathway, stimulation of the Nrf2 signalling pathway, inhibition of NADPH oxidases and COX have been demonstrated for some specific polyphenols [3, 5, 20].

Cyanidin is a flavonoid that belongs to the anthocyanins group. Anthocyanins are water-soluble pigments present in many fruits and vegetables. Some studies demonstrated that oral administration to rats of cyanidin-3-glucoside (C3G) attenuates the inflammatory response through a decrease in the expression of inducible nitric oxide synthase (iNOS) and a suppression of pro inflammatory cytokine production [21].

Chlorogenic acid is a phenolic compound, very abundant in coffee, where it represents 98% of the total phenolic content (Martini et al, 2016). Chlorogenic acid in addition to being present in raw coffee, is also found

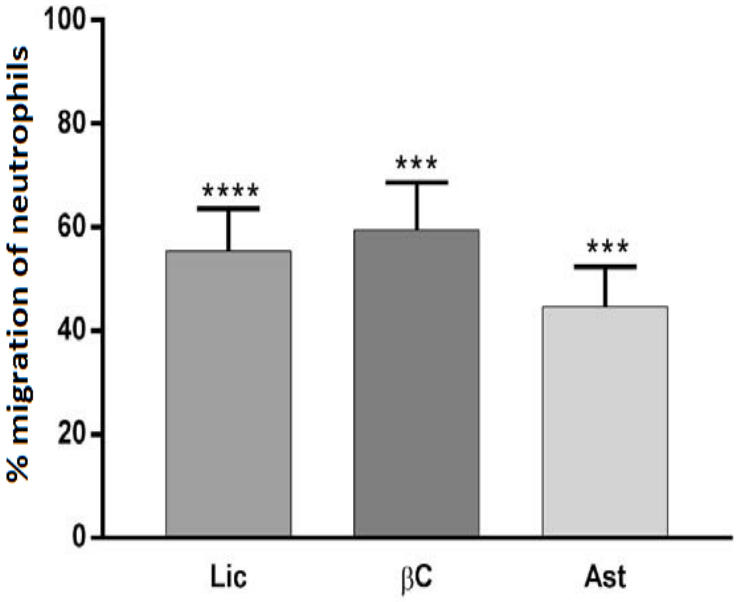

Figure 5. Neutrophil migration in larvae exposed to some carotenoid compounds present in "sofrito": Lycopene 20 μM (Lic), β-Carotene 25 μM (βC) and Astaxanthin 20 μM (Ast).

in many types of seeds and fruits such as sunflower seeds and blueberries. A lower content of chlorogenic acid has also been detected in potatoes, tomatoes, apples, pears and aubergines, but consumption of these sources represents 5 to 10% of the chlorogenic acid from the coffee source. Tajik et al, [22] showed that, in addition to its antioxidant and anti-inflammatory effects, chlorogenic acid is capable of exerting essential functions in the regulation of glucose and lipid metabolism, as well as in the disorders related to diabetes, cardiovascular disease, obesity and cancer.

Carotenoid compounds intake is high in the Mediterranean diet because they are present in multiple highly consumed foods as tomato and carrots. Two of the most abundant bioactive carotenoid pigments are lycopene and β-carotene. The strong antioxidant effects and other beneficial effects in vitro and in vivo of the carotenes are associated with their capacity to act as free radical scavengers. Carotenoids are pigments responsible for the color of fruits, flowers and leaves. Lycopene is lipophilic, red and is present in ripe tomatoes, the orange color of carrots is caused by β-carotene and the pink / red color is due to astaxanthin [23]. Most research on the health effects of tomato intake and tomato products is concentrated in lycopene; its health benefits have been studied especially in the prevention of chronic inflammatory diseases, cancer and cardiovascular problems [24, 25]. Our results (Fig. 5) show a moderate inhibitory activity on the neutrophil migration.

Antioxidant protective effects

In most of the cases, pre-exposure to the studied compounds produced a significant drift of the concentration-response curves of lethality and dysmorphogenesis to higher concentrations of tBOOH (Fig. 6; Table 1). The effect in lethality is in general more evident, in part by the higher variability of the morphologic effects.

It is surprising the lack of effect of resveratrol and EGCG, two of the most reputed health promoting polyphenols. These results could be explained by a limited bioavailability. However, previous results showed that polyphenols are well absorbed by zebrafish embryo from the water media [26]. On the contrary, the reason of the lack of effect could be an excessive concentration that could yield to a pro-oxidant effect.

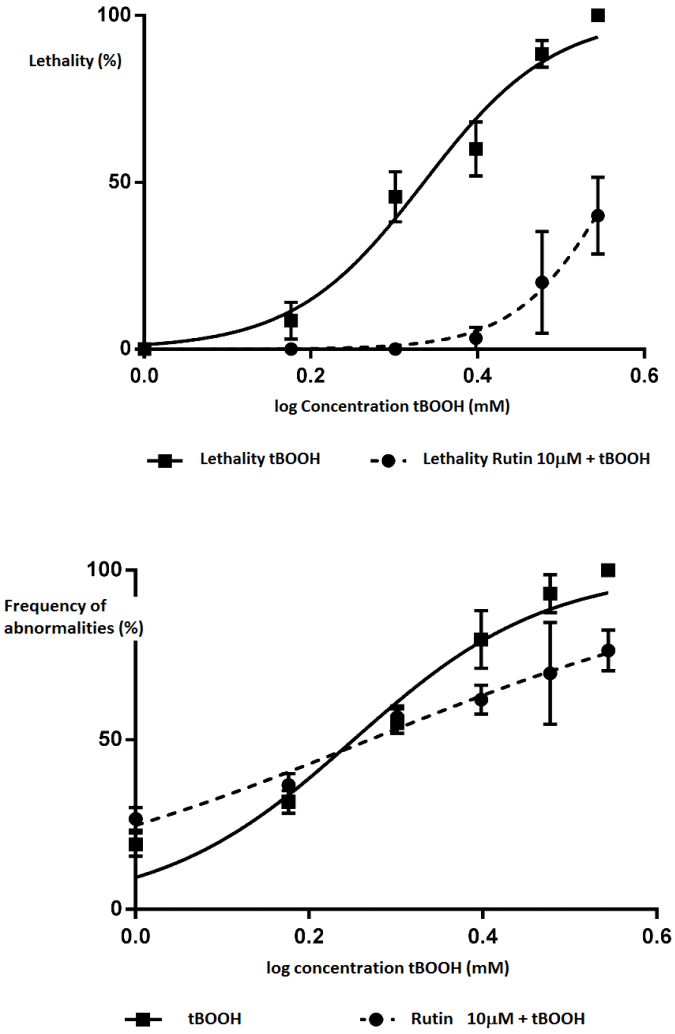

Figure 6. Concentration-response curves of lethality and dysmorphogenesis induced by tBOOH exposure in zebrafish embryo. In the case of pre-exposure to rutin 10 μM, the lethality curve shows a significant shift to the right side, indicating a protective anti-oxidant effect. The effect on dysmorphogenesis is less clear but a significant decrease of the slope is observed.

Table 1. Effect of some polyphenols on the lethality and morphologic abnormalities induced by tBOOH in zebrafish embryos. AOX: antioxidant protective effect; INC: the compound increases the adverse effect; NE: no significant effect observed.

Compound	Lethality			Morphologic abnormalities		
	LC_{50} tBOOH	LC_{50} tBOOH+ compound	Effect	EC_{50} tBOOH	EC_{50} tBOOH + compound	Effect
Naringenin 20 µM	2.17	3.48	AOX	1.78	1.80	NE
Oleuropein 15 µM	2.17	2.65	AOX	1.78	1.85	NE
Rutin 10 µM	2.17	3.68	AOX	1.78	1.86	AOX
Cyanidin 20 µM	2.17	3.23	AOX	1.78	1.54	INC
Clorogenic Ac. 20 µM	2.17	2.59	AOX	1.78	1.90	AOX
Apigenin 10 µM	2.17	3.35	AOX	1.78	2.00	AOX
Resveratrol 20 µM	2.71	2.61	NE	2.37	1.86	INC
Curcumin 10 µM	2.71	3.20	AOX	2.37	3.02	AOX
EGCG 20 µM	2.71	2.47	NE	2.37	2.69	NE

3. Conclusions

A method for the measurement of neutrophil migration in a whole organism in zebrafish larvae has been adapted and optimized. The method has been proven reliable and repetitive, although due to the variability of the individual response, the use of a large number of larvae and at least three replicates with different spawn are required. The quantification of migration, by means of image analysis, is reproducible and objective. By means of this established model, the anti-inflammatory activity of a selection of polyphenols and carotenoids of the Mediterranean diet has been studied.

A method for the assessment of antioxidant activity in a whole organism has been developed and applied to a series of natural polyphenols. The quantification of lethality and dysmorphogenesis is easy and reproducible. The protection against the in vivo effects of oxidative stress can be assessed independently of the actual mechanism of "antioxidants" action.

These methods can be applied to food extracts and can be useful as screening tests for the identification of fractions and individual compounds with potential anti-inflammatory and antioxidant activity. Additional work is necessary in order to assess the concentration-effect relationships of the anti-inflammatory and antioxidant activities of natural food compounds.

Acknowledgements

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