





Original Research

Genotoxicity Assessment of Quinoin, a Ribosome Inactivating Protein from Quinoa Seeds, in the Teleost *Danio rerio*

Filomena Mottola¹, Sara Ragucci¹, Maria Carannante¹, Nicola Landi^{1,2},
Rosaria Scudiero³, Antimo Di Maro^{1,*}, Lucia Rocco^{1,*}¹Department of Environmental, Biological and Pharmaceutical Sciences and Technologies (DiSTABiF), University of Campania 'Luigi Vanvitelli', 81100 Caserta, Italy²Institute of Crystallography, National Research Council of Italy, 81100 Caserta, Italy³Department of Biology, University Federico II, 80126 Napoli, Italy*Correspondence: antimo.dimaro@unicampania.it (Antimo Di Maro); lucia.rocco@unicampania.it (Lucia Rocco)

†These authors contributed equally.

Academic Editor: Gheyath K. Nasrallah

Submitted: 27 July 2023 Revised: 7 September 2023 Accepted: 29 December 2023 Published: 4 February 2024

Abstract

Background: Ribosome inactivating proteins (RIPs) are N-glycosylases found in various plants that are able to specifically and irreversibly inhibit protein translation, thereby leading to cell death. Their cytotoxic properties have attracted attention in the medical field in the context of developing new anticancer therapies. Quinoin is a novel toxic enzyme obtained from quinoa seeds and classified as a type 1 RIP (*Chenopodium quinoa* Willd.). Recently, quinoin was found to be cytotoxic to normal fibroblasts and keratinocytes *in vitro*, as well as to several tumor cell lines. **Methods:** The aim of this study was to evaluate the *in vitro* and *in vivo* genotoxicity of quinoin in a zebrafish model. We evaluated its ability to induce DNA fragmentation, genomic instability, and reactive oxygen species (ROS) generation by means of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) reaction, randomly amplified polymorphic DNA (RAPD) Polymerase Chain Reaction (PCR) technique, and dichlorofluorescein (DCF) assay, respectively. **Results:** Quinoin was found to cause genomic damage in zebrafish, as shown by DNA fragmentation, polymorphic variations leading to genomic instability, and oxidative stress. Interestingly, longer quinoin treatment caused less damage than shorter treatments. **Conclusions:** This study demonstrated ROS-mediated genotoxicity of quinoin toward the zebrafish genome. The reduced damage observed after longer quinoin treatment could indicate the activation of detoxification mechanisms, activation of repair mechanisms, or the loss of protein activity due to enzymatic digestion. In order to clarify the genotoxic actions of quinoin, further investigations of the response pathways to DNA damage are needed. Overall, the ability of quinoin to cause breaks and instability in DNA, together with its clear cytotoxicity, make it an interesting candidate for the development of new drugs for cancer treatment.

Keywords: *Chenopodium quinoa* Willd.; DNA damage; genotoxic enzyme; N- β -glycosylase; oxidative stress; zebrafish

1. Introduction

Edible parts of plants, such as seeds, fruits, roots and leaves, are important for human nutrition because they contain metabolites and macromolecules that are essential for growth and health, including carbohydrates, sugars, vitamins and proteins [1]. However, different plant tissues also contain harmful or toxic substances that are synthesized as defense mechanisms against biotic attack by insects, fungi and viruses [2], and against abiotic environmental stresses such as radiation and heat stress [3]. Most of these toxic substances belong to several classes of chemical compounds, including phytates, tannins, alkaloids, oxalates, saponins, cyanides and raffinose oligosaccharides, or to protein families such as protease inhibitors, lectins, α -amylase inhibitors and lipase inhibitors. Together, they are generally referred to as anti-nutritional factors (ANFs) [4,5].

Other known plant toxic proteins are ribosome inactivating proteins (RIPs) found in angiosperms. RIPs are en-

zymes (EC: 3.2.2.22) endowed with rRNA N-glycosylases activity. They are necessary for the removal of a specific adenine (A4324 in rats) in the Sarcin Ricin Loop (SRL) of 28S rRNA, which is involved in the interaction between ribosomes and elongation factors. This irreversible rRNA damage leads to the inhibition of translation and cell death by the apoptotic pathway [6,7].

Interest in RIPs has increased in recent years due to their use in experimental studies as the toxic moiety of immunoconjugates that target various human pathologies, including cancer [8]. The anti-tumor potential of RIPs has been demonstrated in clinical studies with immunotoxins [9]. For example, saporin-S6 type 1 RIP, isolated from the seeds of *Saponaria officinalis* L., has been used to construct conjugates against numerous targets and shows good efficacy in hematological cancer models [10]. Stenodactylin, purified from the caudex of *Adenia stenodactyla* Harms, is a potent type 2 RIP that induces multiple cell death pathways, mainly involving apoptosis, but also necroptosis and



free radical production in a neuroblastoma cell line [11]. Kirkiin, purified from the caudex of *Adenia kirkii* (Mast.) Engl., is a type 2 RIP that can completely inhibit protein synthesis and induce cell death by apoptosis at very low doses in a human neuroblastoma cell line [12].

Recently, a novel type 1 RIP called quinoin was purified from quinoa seeds and extensively characterized [13]. The quinoa plant (*Chenopodium quinoa* Willd.) is native to the Andes of South America [14]. Quinoa seeds, known as the 'Golden Grain', are rich in raw proteins and contain all of the essential amino acids, dietary fibers, B vitamins, and dietary minerals. The consumption of quinoa seeds has therefore continued to increase, together with the production of its derivatives such as bread, pasta, biscuits and cakes [15].

Nevertheless, quinoa seeds contain ANFs including saponins, phytic acid, oxalates, tannins, and trypsin inhibitors [16,17]. In addition to cooking, quinoa seeds are subjected to other food processing methods such as extrusion, roasting, or mechanical abrasion, all of which remove ANFs [17,18]. Aside from its health benefits, this pseudocereal could also be used in the medical field for cancer treatment, given the presence of quinoin.

The type 1 RIP quinoin (~29-kDa) can inhibit protein synthesis *in vitro*, shows remarkable thermostability (T_m ~68.2 °C), and is partially resistant to *in vitro* digestion systems [13]. Furthermore, quinoin is cytotoxic towards BJ-5ta (human fibroblasts), HaCaT (human keratinocytes), and several tumor cell lines in a dose- and time-dependent manner [13,19]. As with most RIPs, quinoin can also depurinate DNA to cause genotoxic damage and subsequent apoptosis [20]. Quinoin is thus a novel toxin enzyme found in quinoa seeds (~3.0 mg/100 g of seeds) and also in smaller amounts in the sprouts [13].

The aim of the present study was to evaluate the *in vitro* and *in vivo* acute genotoxicity of quinoin, thereby improving our understanding of the mechanism of action of RIPs. For this purpose, we used the zebrafish model. This represents a valid genotoxicity model to replace mammalian models, since it shares approximately 75% of its genome with humans. Moreover, zebrafish show rapid development, allowing the toxic effects of a given substance to be monitored over a short exposure time [21,22].

DNA fragmentation and Genomic Template Stability (GTS) were assessed in zebrafish blood cells exposed *in vitro* to different amounts of quinoin for different times, as well as in blood cells taken from zebrafish after intraperitoneal administration of different amounts of quinoin for several days. DNA damage is reported to be secondary to oxidative stress, and reactive oxygen species (ROS) have been identified as the main mediators of RIP-induced cell death [23]. We therefore estimated the level of ROS in exposed blood cells in order to correlate oxidative stress with DNA damage caused by quinoin.

This research provides new evidence for the harmful actions of quinoin in causing genomic and subsequent cellular damage, thus highlighting its potential incorporation into immunotoxins for the therapeutic targeting of cancer.

2. Materials and Methods

2.1 Chemicals and Reagents for Protein Purification

Chemicals for chromatography were obtained as previously reported [13], while standard reagents for protein purification and analysis were purchased from Sigma-Aldrich Solutions (Merck Life Science, Milan, Italy).

2.2 Protein Purification

The purification of quinoin for *in vitro* and *in vivo* biological assays was performed as previously reported [13]. Briefly, a crude extract of quinoa seeds was acidified with glacial acetic acid and the soluble protein was fractionated according to differences in protein size (gel filtration chromatography) and charge characteristics (ion exchange chromatography). The protein obtained was first dialyzed against deionized water, then freeze-dried and stored at -20 °C. All general methods used for the analytical characterization of quinoin, such as Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), Reverse Phase High Performance Liquid Chromatography (RP-HPLC), and measurement of the protein concentration by colorimetric assay (BCA assay), were carried out as previously described [24,25].

2.3 Conditions for Zebrafish Growth

Adult zebrafish (*Danio rerio*) were maintained at the Department of Biology, University of Naples Federico II. The handling of animals and the experimental procedures were in accordance with the local regulation and international guidelines for the use and care of laboratory animals [26] and approved by the Italian Ministry of Health (Permit Number: 529/2019-PR). Efforts were made to minimize animal suffering and the number of specimens used. The zebrafish were reared in an aquarium tank at a temperature of between 24 and 29 °C, with a photoperiod of 10 hours dark and 14 hours light, and a pH of ~7.2 to mimic the conditions of their natural habitat [27].

2.4 In Vitro Experimental Design

Each organism was anesthetized with ethyl 3-aminobenzoate methane sulfonate (MS-222, Sigma Aldrich, Darmstadt, Germany) to minimize suffering. Blood was collected from the gills of 10 zebrafish using a heparinized syringe. In order to obtain a sufficient number of cells for all tests, a pool of blood cells from different specimens was subdivided into aliquots, with each containing different final concentrations of quinoin (1, 5 and 10 µg/µL). Cells were exposed to quinoin for 30, 60 and 90 minutes, after which the genotoxicity tests were performed.

2.5 *In Vivo* Experimental Design

After 2 weeks of acclimation, 45 zebrafish were sorted into 5 L tanks as follows: 12 specimens for each group were divided into three tanks. The remaining specimens were sorted into one other tank and injected with 1 μ L of sterile water to serve as a negative control. Following zebrafish anesthesia as reported above, inoculation with 1, 5 and 10 μ g quinoin was performed by intraperitoneal injection into the abdominal cavity posterior to the pelvic girdle using a 10 μ L 35G blunt steel Hamilton-type micro needle (SYR 10 μ L – 1701 RN, WO: 1900029358). After administration of the quinoin, zebrafish specimens were transferred to suitably prepared tanks and their swimming movements were monitored to ensure they were not stressed. At 15 and 30 days after injection, approximately 30 μ L of blood was collected from the gills of each fish with a heparinized syringe and the tests were performed.

2.6 DNA Fragmentation

DNA fragmentation was determined using an *in situ* cell death detection kit (Roche Diagnostics, Basel, Switzerland) as previously reported [28]. Briefly, blood cells were mixed with phosphate-buffered saline (1 \times PBS, Thermo Fisher Scientific, Waltham, MO, USA) and the pellet was isolated by centrifugation at 2000 revolutions per minute (rpm) for 10 minutes. A subsequent cell suspension in 1 \times PBS was applied to slides and fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 1 hour. After permeabilization for 2 minutes in 0.1 g sodium citrate and 0.1% Triton X-100 solution, slides were incubated with the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) reaction mixture for 1 hour at 37 $^{\circ}$ C, washed in 1 \times PBS, and then stained with a 4',6-diamidino-2-phenylindole contrast solution (DAPI, Sigma-Aldrich, St. Louis, MO, USA). For each experimental condition, approximately 300 cells per slide (in triplicate) were examined under a fluorescence microscope (Nikon Eclipse E-600, Minato, Tokyo, Japan) equipped with 330–380 nm BP and 420 nm LP filters. The DNA fragmentation index (DFI) was calculated as the percentage of green nuclei with fragmented DNA out of the total blue nuclei with intact DNA.

2.7 Evaluation of Genomic Template Stability

DNA was isolated from zebrafish blood cells using the High Pure PCR Template Preparation Kit (ROCHE Diagnostics). Genomic DNA amplification was performed using primer 6 (5'-CCCGTCAGCA-3') and the amplification scheme reported previously [28]. The Random Amplification of Polymorphic DNA- Polymerase Chain Reaction (RAPD-PCR) profiles obtained were used to calculate the percentage of GTS (%) as follows: $GTS = (1 - a/n) \times 100$, where a is the mean number of polymorphic bands (appearance and disappearance compared to controls) in the treated samples, and n is the total number of bands in the untreated samples.

2.8 Detection of Reactive Oxygen Species

In order to calculate the intracellular percentage of ROS, 13 μ M of 2',7'-dichlorodihydrofluorescein diacetate (DCFH₂-DA) (Sigma Aldrich®, Darmstadt, Germany) was incubated for 30 minutes at 37 $^{\circ}$ C in the dark with a 1x PBS suspension of blood cells from each study group. After incubation, excess unreacted DCFH₂-DA was removed by centrifugation at 2000 rpm for 10 minutes. The cells were counterstained with DAPI solution for 5 minutes and then placed on a slide for observation under a fluorescence microscope. Approximately 300 cells per slide (in triplicate) were analyzed for each experimental condition. Intracellular levels of ROS were quantified as the percentage of cells with green fluorescence out of the total cells with blue nuclei [29].

2.9 Statistical Analysis

The percentages of DNA fragmentation, genome stability and intracellular ROS were expressed as the mean \pm standard deviation (SD). U-test (Mann-Witney-Wilcoxon test) and *t*-test, as nonparametric tests, were used for statistical evaluation using GraphPad Prism 10 (GraphPad Software Inc., San Diego, CA, USA). All tests were performed in triplicate. The effect was considered significant with a *p*-value ≤ 0.05 .

3. Results

3.1 Zebrafish Survival and Physical Condition after Quinoin Inoculation

All zebrafish specimens survived after quinoin injection. No signs of suffering or changes in physical condition or movement were observed during the 30 days following injection of the three quinoin doses.

3.2 *In Vitro* and *In Vivo* DNA Fragmentation

Cellular DNA fragmentation as observed with the TUNEL technique is shown in Fig. 1. DNA fragmentation in zebrafish blood cells increased significantly following *in vitro* exposure to quinoin (Fig. 2). This was observed for all treatment times, regardless of the quinoin concentration used. However, DNA fragmentation decreased with increasing exposure time, with values ranging from $19.25 \pm 2.5\%$ to $10.5 \pm 1\%$.

A time-dependent decrease in the percent DFI was also observed after *in vivo* treatment with quinoin (Fig. 3). Administration of 1, 5 and 10 μ g quinoin resulted in DFI percentages of 39.75 ± 1.7 , 57.5 ± 2.08 and 57.4 ± 1.3 , respectively, 15 days injection. A lower DFI percentage was observed in specimens 30 days after injection compared to 15 days after injection. Specifically, the DFI percentages after administration of 1, 5 and 10 μ g quinoin were 30.2 ± 1.5 , 27.85 ± 1 and 37.7 ± 1.5 , respectively.

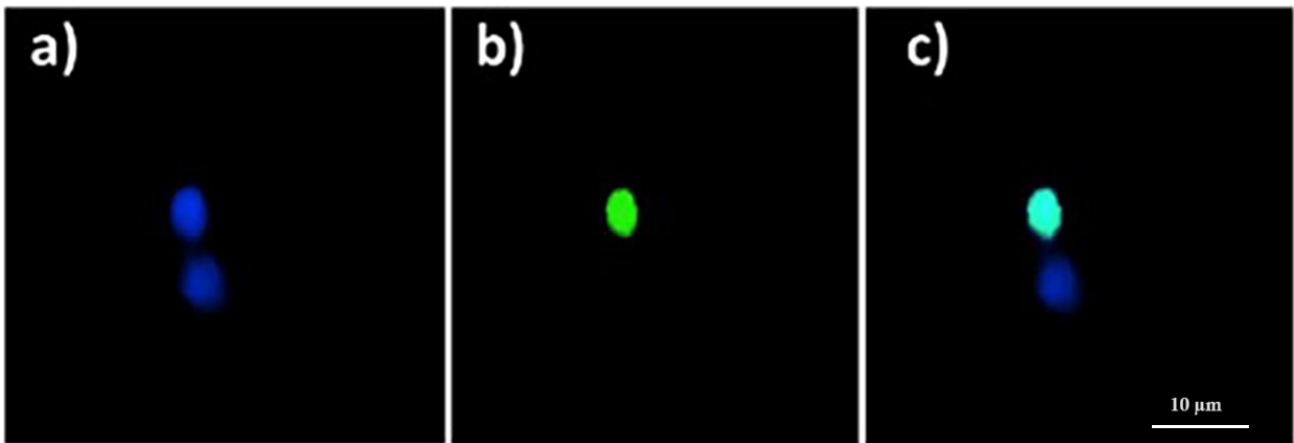


Fig. 1. Quinoin-treated zebrafish erythrocytes showing fragmented DNA (green nuclei), and no DNA fragmentation (blue nuclei), as observed with the Terminal deoxynucleotidyl transferase (TdT) 2'-Deoxyuridine, 5'-Triphosphate (dUTP) Nick-End Labeling (TUNEL) technique. (a) DAPI. (b) Fluorescein isothiocyanate (FITC). (c) merge. DAPI, 4',6-diamidino-2-phenylindole.

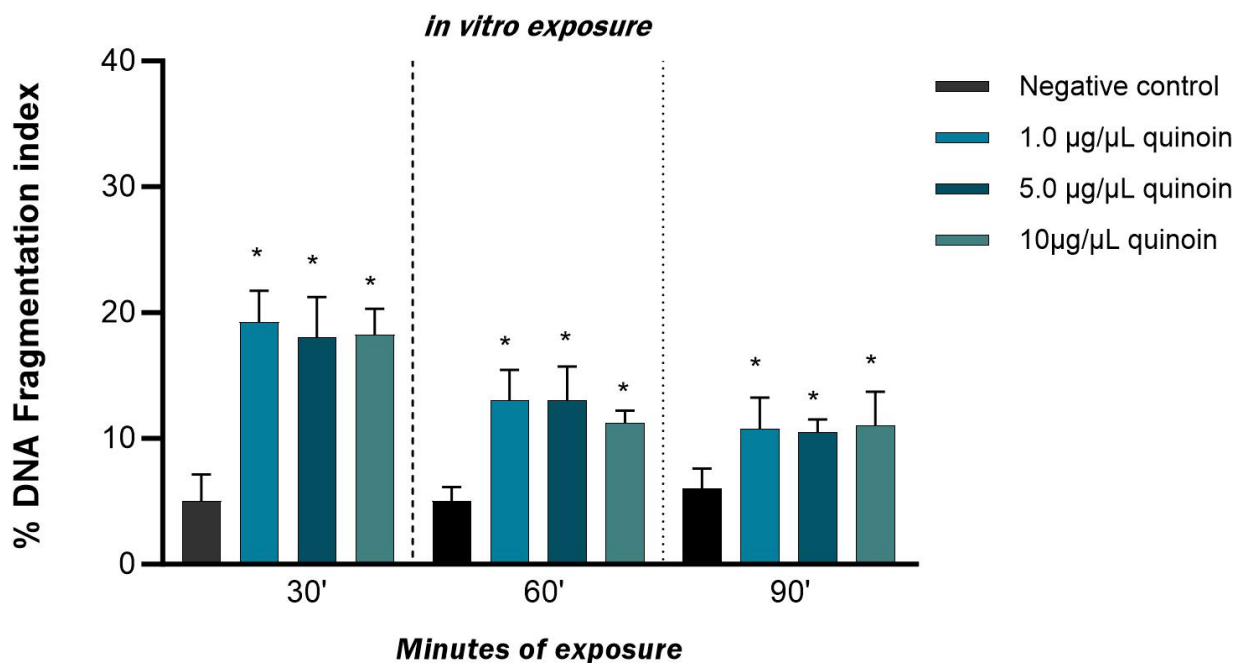


Fig. 2. Percentage of DNA fragmentation observed in zebrafish blood cells after 30, 60 and 90 minutes of *in vitro* exposure to different quinoin concentrations. * $p < 0.05$ compared to control.

3.3 *In Vivo* and *In Vitro* Polymorphic Variation

A quinoin concentration of 1 μg/μL induced a single polymorphic variation (lost band) after 30, 60 and 90 minutes of *in vitro* treatment (Table 1, Fig. 4). At 5 μg/μL quinoin, three variations were observed after 30 minutes of treatment, and one variation at each of 60 and 90 minutes exposure. At the highest concentration of quinoin (10 μg/μL), three variations were observed after 30 minutes of treatment, and one variant band at each of 60 and 90 minutes.

The electrophoretic profile of zebrafish DNA 15 days after 1 μg quinoin injection revealed three variations, whereas 30 days after administration of 5 and 10 μg quinoin only one variation was found, regardless of the amount of quinoin administered (Table 2; Fig. 5).

3.4 *In Vivo* and *In Vitro* %GTS

The polymorphic profiles obtained with the RAPD-PCR technique were used to calculate the genomic stability of the template. Genome stability was reduced after *in vitro*

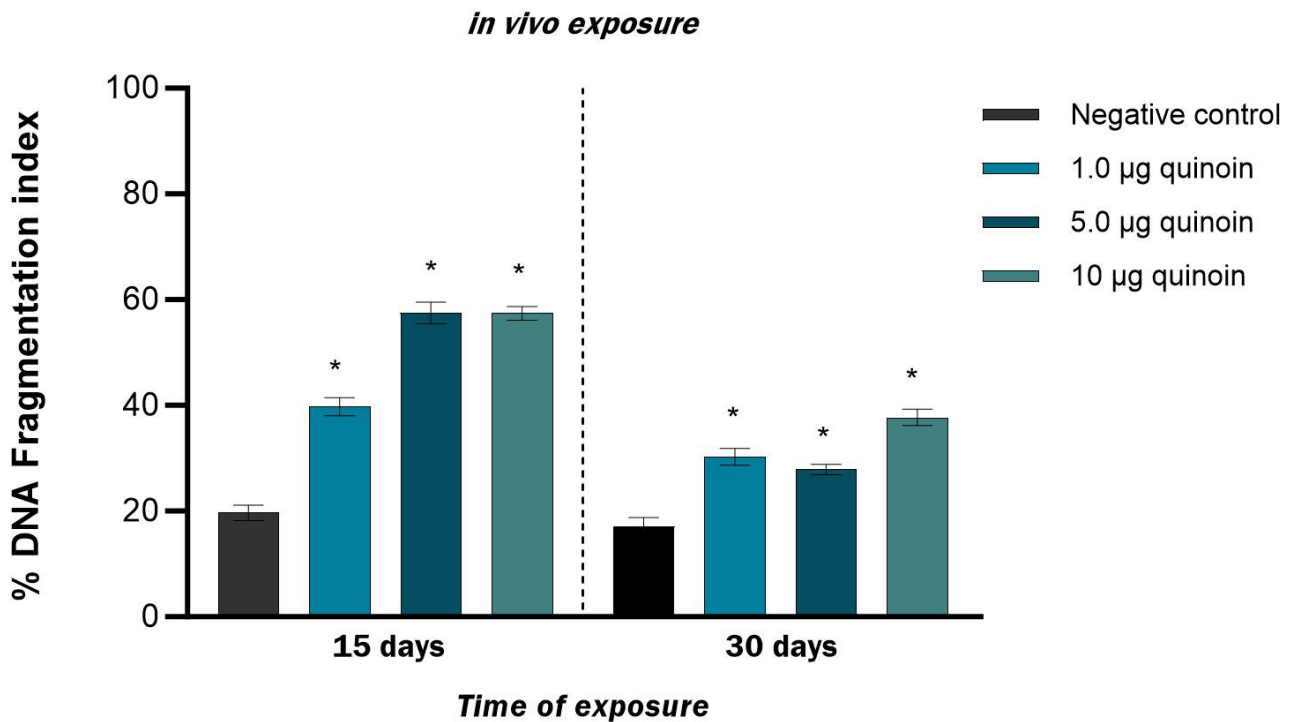


Fig. 3. Percentage of DNA fragmentation observed in zebrafish blood cells after 15 and 30 days of *in vivo* injection of different amounts of quinoin. * $p < 0.05$ compared to control.

Table 1. Randomly amplified polymorphic DNA (RAPD) - Polymerase Chain Reaction (PCR) profiles of zebrafish DNA following 30, 60 and 90 minutes of *in vitro* treatment with different quinoin concentrations.

Quinoin concentration	Minutes of exposure	Lost Bands	Gained Bands
1 µg/µL	30	510 bp	-
	60	510 bp	-
	90	-	350 bp
5 µg/µL	30	510 bp	350 bp 780 bp
	60	-	350 bp
	90	-	780 bp
10 µg/µL	30	510 bp	350 bp 820 bp
	60	-	420 bp
	90	-	420 bp

Control bands: 200 bp, 210 bp, 300 bp, 400 bp, 500 bp, 510 bp, 600 bp, 800 bp, 1000 bp.

treatment with 1, 5 and 10 µg/µL quinoin for 30, 60 and 90 minutes, with a maximum reduction of 23% observed after 30 minutes of 10 µg/µL quinoin (Fig. 6).

15 days after *in vivo* administration of 1 µg quinoin reduced genome stability by 60%. Administration of 1, 5 and 10 µg quinoin reduced genome stability 30 days after injection by an average of approximately 20% (Fig. 7).

3.5 Intracellular Detectable ROS after *in Vitro* and *in Vivo* Treatment

Fig. 8 shows intracellular ROS, as seen with the 2',7'-dichlorodihydrofluorescein (DCF) assay. A time-dependent decrease in the percentage of cells with detectable ROS was observed after quinoin treatment, from approximately 14% for the 30 minutes treatment to 12% and 10% for 60 and 90 minutes treatment, respectively (Fig. 9).

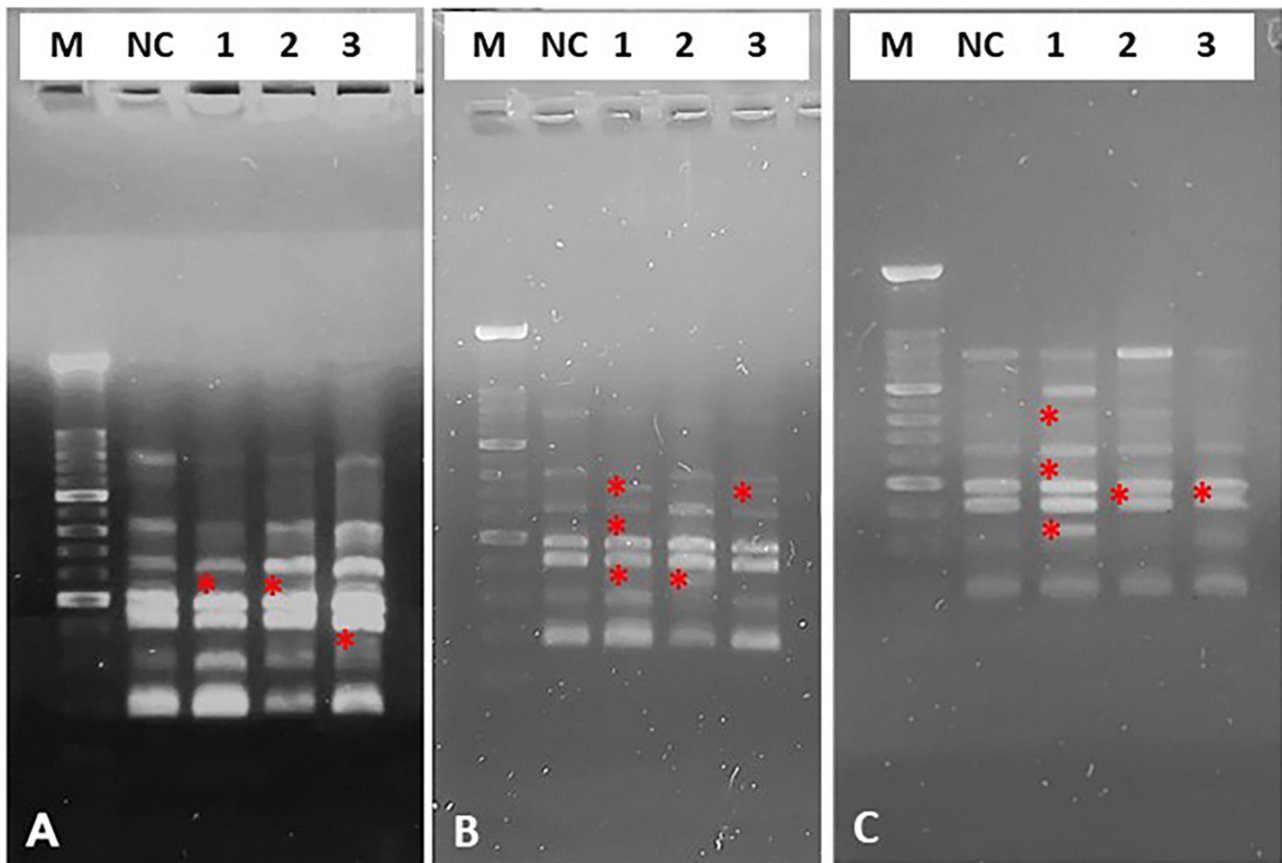


Fig. 4. Representative image of electrophoretic profiles of zebrafish DNA following 30 (A), 60 (B) and 90 (C) minutes of *in vitro* treatment with different quinoin. M, 100 bp DNA Marker; NC, negative control; 1, 1 µg/µL quinoin; 2, 5 µg/µL quinoin; 3, 10 µg/µL quinoin. * polymorphic bands.

Table 2. RAPD-PCR profiles of zebrafish DNA following 15 and 30 days after injection of different amounts of quinoin.

Quinoin	Days of exposure	Lost Bands	Gained Bands
1 µg	15	-	280, 320, 550 bp
	30	520 bp	-
5 µg	15	-	280 bp
	30	520 bp	-
10 µg	15	-	280bp
	30	520bp	-

Control bands: 200 bp, 220 bp, 300 bp, 400 bp, 520 bp.

A significantly higher percentage of cells with intracellular ROS was found 15 days after administration of 1, 5 and 10 µg of quinoin, with a maximum of $26.2 \pm 0.5\%$ for 5 µg quinoin. For all amounts of quinoin, a slight decrease in the percentage of cells with intracellular ROS was seen 30 days after injection (Fig. 10).

4. Discussion

RIPs are toxic enzymes that can damage ribosomes and inhibit protein synthesis. They have attracted much

attention in the medical and agriculture fields due to their antitumor, antifungal, antibacterial, antiviral and insecticidal activities [30]. RIPs are mainly found in the edible part of plants, such as seeds, leaves and roots. However, some RIPs are not harmful if the plant source is consumed after appropriate cooking. This is because they are denatured by heating, and hence their toxicity is reduced and/or eliminated [31]. Nevertheless, RIP activity has been detected in some plant tissues that are eaten raw, such as *Allium cepa* L., *Spinacia oleracea* L., *Apium graveolens* L., and *Daucus carota* L., or the seeds of *Lycopersicon esculentum* L. [32]. The low concentration of RIPs in these products is likely to make them safe for consumption.

Quinoa is one of the plant products that is eaten raw or cooked, and was recently included in the Mediterranean diet for its antioxidant and anti-inflammatory properties [33].

Quinoa is a pseudo-cereal belonging to the Chenopodiaceae family. It is an excellent source of nutrients and a valid alternative to commonly used cereals and carbohydrates, as it is rich in fiber, minerals, high quality proteins, vitamins (B, C and E group) and essential fatty acids. Quinoa is also an ideal food for coeliacs because it is gluten-free [17]. The consumption of quinoa seeds has been shown

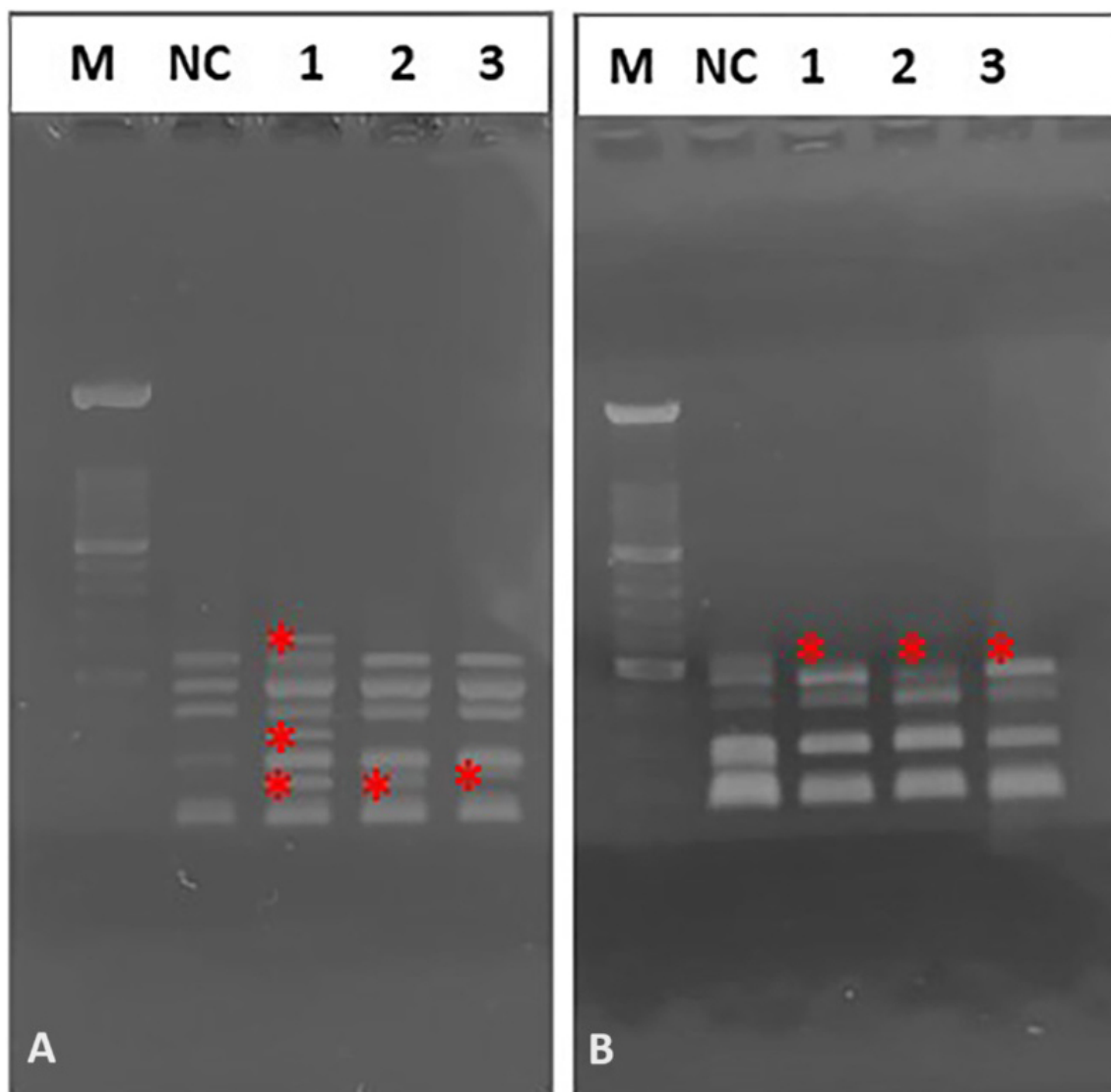


Fig. 5. Representative image of electrophoretic profiles of zebrafish DNA following 15 (A), and 30 (B) days after administration of different quinoin amount. M, 100 bp DNA Marker; NC, negative control; 1, 1 µg/µL quinoin; 2, 5 µg/µL quinoin; 3, 10 µg/µL quinoin. * polymorphic bands.

to increase intestinal permeability, improve the absorption of certain proteins, and produce anti-inflammatory effects [34]. However, quinoa also contains ANFs that can react with nutrients and interfere with their absorption [35]. It is particularly rich in saponins, which may reduce its potential benefits.

Quinoin is a novel type 1 RIP that was recently extracted from quinoa seeds. As with other RIPs, this enzyme acts by removing a specific adenine in 28S ribosomal RNA (rRNA), thereby blocking translocation and inducing cell death [13]. Its cytotoxicity has been demonstrated in both fibroblasts and glioblastoma tumor cell lines, which show clear changes in cell morphology and reduced cell growth following exposure to quinoin [19].

Although RIPs were previously thought to act only on rRNA, it is now known that many can also remove adenine

bases from naked RNAs and from DNA. These genotoxins are capable of causing damage to nuclear DNA, leading to apoptosis [36].

In light of previous results showing a cytotoxic effect of quinoin in various experimental models, the aim of the present work was to evaluate the potential acute genotoxic effects of this enzyme *in vitro* and *in vivo* using the well-known zebrafish model system [21].

To this end, DNA fragmentation, genome stability interference and oxidative stress were assessed in zebrafish blood cells after *in vitro* exposure to different quinoin concentrations for different times and following different days after *in vivo* administration of several quinoin amounts.

The results obtained using the TUNEL reaction were consistent with a previous report in which quinoin was described as able to induce programmed cell death [19]. In-

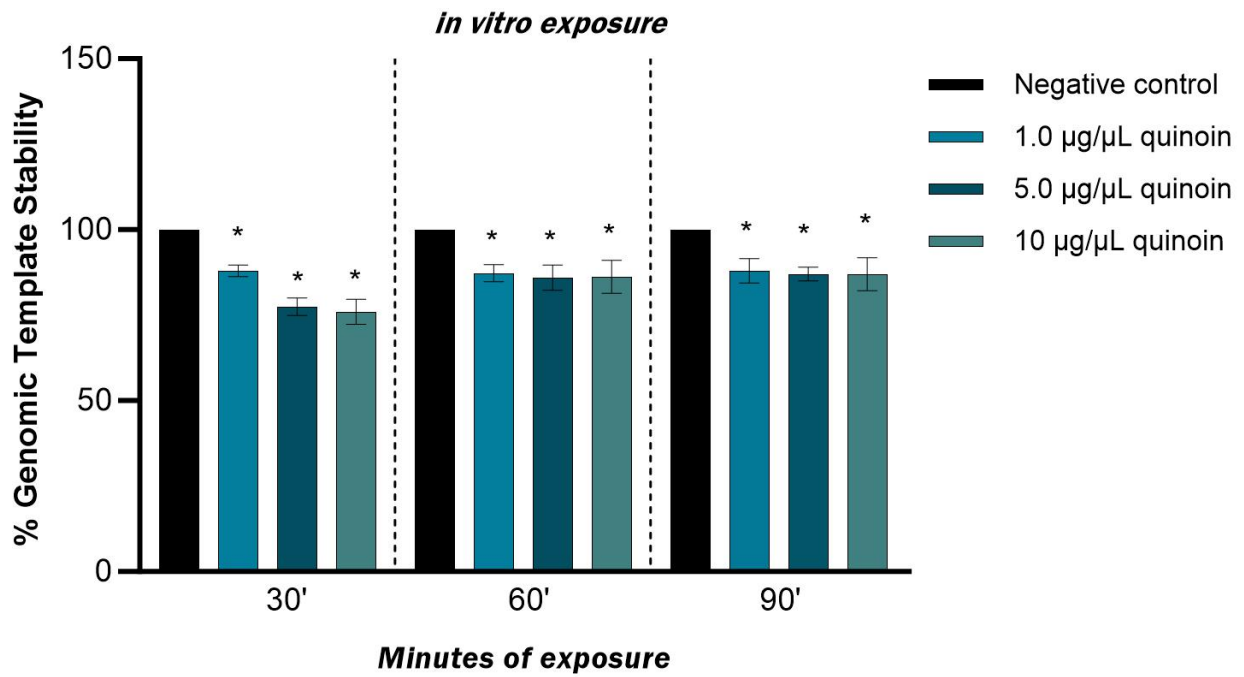


Fig. 6. Percentage of genomic template stability observed in zebrafish blood cells after 30, 60 and 90 minutes of *in vitro* exposure to different quinoin concentrations. * $p < 0.05$ compared to control.

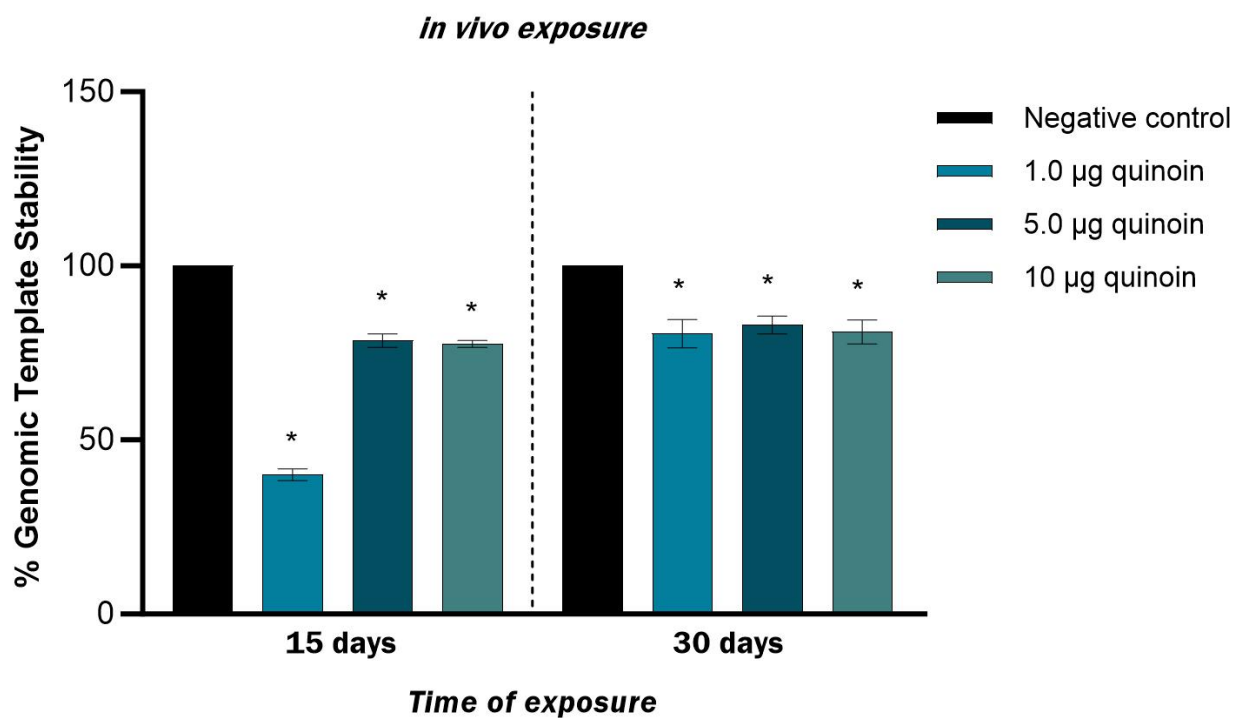


Fig. 7. Percentage of genomic template stability observed in zebrafish blood cells 15 and 30 days after *in vivo* injection of different amounts of quinoin. * $p < 0.05$ compared to control.

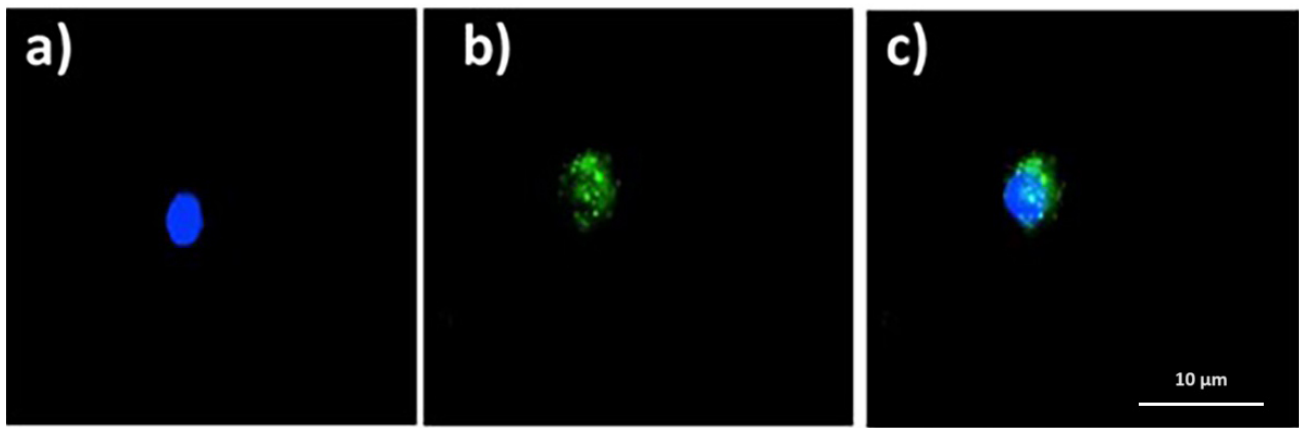


Fig. 8. Quinoin-treated zebrafish erythrocytes with intracellular ROS (green fluorescence) after DCF assay. (a) DAPI. (b) FITC. (c) Merge. ROS, reactive oxygen species; DCF, 2',7'-dichlorodihydrofluorescein.

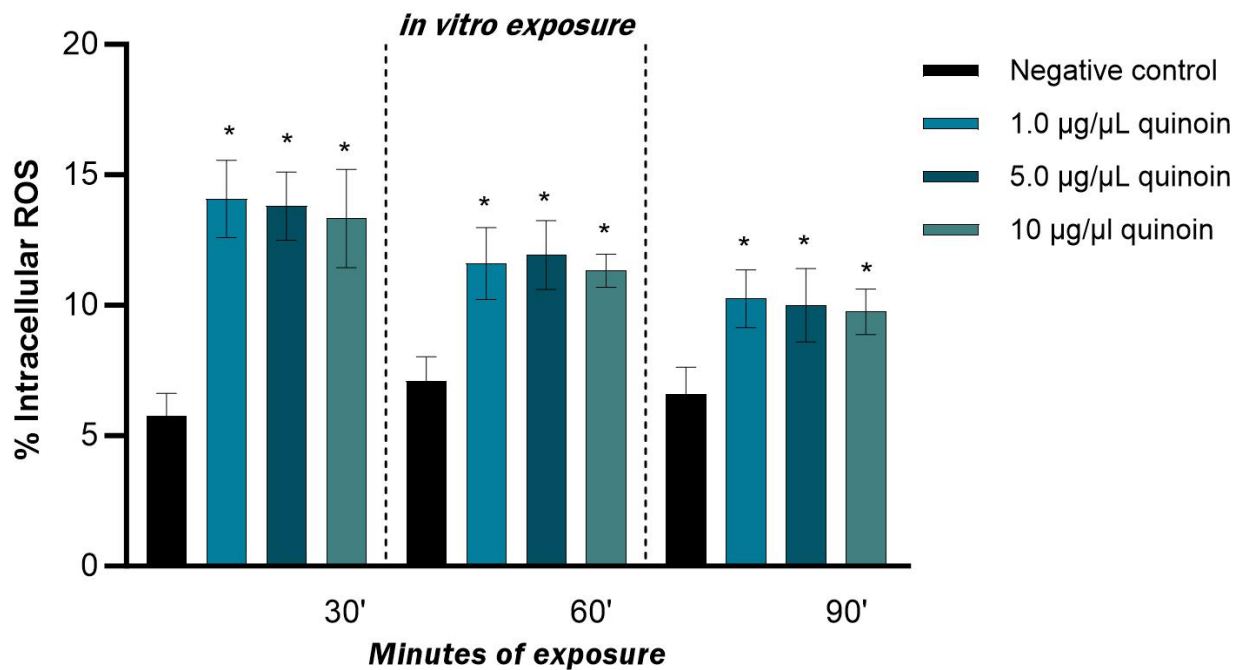


Fig. 9. Percentage of intracellular ROS observed in zebrafish blood cells after 30, 60 and 90 minutes of *in vitro* exposure to different quinoin concentrations. * $p < 0.05$ compared to control.

deed, DNA fragmentation is the main feature of apoptosis and is used as a specific marker of this process [37]. Compared to the negative control, the percent of DNA fragmentation was significantly higher in all samples treated with quinoin. Similarly, genomic template stability was reduced after quinoin treatment, as observed by the appearance of polymorphic bands. These results indicate that quinoin can induce damage at the genomic DNA level, either directly or indirectly [38], and also confirming that it can act on rRNA as well as DNA.

Many genotoxic substances are known to indirectly affect the cell nucleus through the production of free rad-

icals [39]. Indeed, high percentages of intracellular ROS were detected here after both *in vivo* and *in vitro* exposure to quinoin, thus unequivocally indicating the presence of oxidative stress-mediated DNA damage.

Based on experimental evidence in the literature, it was hypothesized that quinoin acts on zebrafish cells by slowing down normal mitochondrial function. It has been reported that abrin, a RIP from *Abrus precaricus* L., induces the shutdown of antioxidant protein-1 (AOP-1). AOP-1 normally activates antioxidant factors to help maintain the redox balance in cells. Blocking this system causes the release of cytochrome C and activation of the apoptotic path-

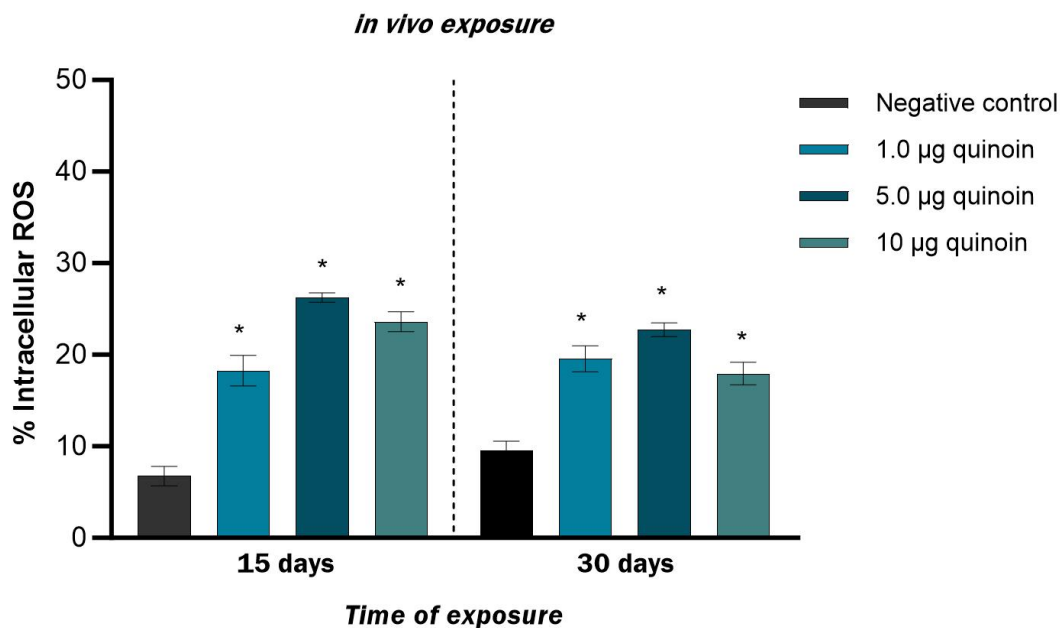


Fig. 10. Percentage of intracellular ROS observed in zebrafish blood cells 15 and 30 days after *in vivo* administration of different amounts of quinoin. * $p < 0.05$ compared to control.

way, with ROS identified as mediators of AOP-1-induced apoptosis [40]. Although the mechanism by which quinoin leads to oxidative stress has not yet been elucidated, its ability to cause ROS-mediated DNA damage has been demonstrated.

It is interesting to note that the percentage of damage was fairly consistent between the different experimental groups, except for the lowest amount of toxin used (1 µg). In the *in vivo* experiments, 1 µg of quinoin resulted in more instability of the genomic template compared to the higher amounts (5 µg and 10 µg). Alterations in RAPD profiles are due to induced molecular events such as genomic rearrangements, point mutations, deletions and insertions. These can be transient and can also be restored by DNA repair mechanisms, hence they do not always lead to apoptotic processes. Moreover, polymorphic variations do not always result in DNA fragmentation, as this can depend on the type of mutagenic substance, the cell type, and the type of induced polymorphism (appearance or disappearance) [29,41,42].

In support of the hypothesis for instant and transitory changes in RAPD profiles, both *in vitro* and *in vivo* experiments showed that longer quinoin exposure times resulted in less damage than fewer time of *in vitro* exposure, in the same way, the damage found 30 days after injection was reduced compared to 15 days after injection. The reduction in damage with prolonged *in vitro* treatment and many days after administration could indicate cellular adaptation and activation of endogenous repair mechanisms, but may also be due to a reduction in protein N-glycosylase activity.

Although the above hypothesis of cellular adaptation needs to be confirmed with further studies, the weak resistance of quinoin was recently demonstrated using an *in vitro* pepsin-trypsin (PT) enzyme system [13]. The results showed that most of the toxin was hydrolyzed in the presence of pepsin and trypsin. However, our study does not involve oral administration and therefore digestion in the gastro-intestinal tract cannot be assumed.

Considering, however, that from the aforementioned study it emerges that a small percentage of quinoin remained active even in the presence of hydrolytic enzymes, it is still advisable to treat quinoa prior to consumption in order to inactivate/reduce ANFs. However, our study does not support any obvious risk to health from quinoa, given that the relative quantities of quinoin administered to zebrafish were significantly higher than those found in 100 g of quinoa consumed by humans [13]. Furthermore, lower amounts of quinoin did not cause significant damage in the zebrafish genome (data not shown).

Although less damage was observed with prolonged *in vitro* treatment by quinoin and after many days from quinoin *in vivo* administration, genotoxicity remained evident at all times. The proven cytotoxicity of quinoin in various tumor lines *in vitro* could be the basis for its use in the development of new drugs for cancer therapy.

So far, however, the clinical translation of RIPs for cancer treatment is still a long-term goal. The main difficulty lies with gaining entry into the cell due to vascular, intra-tumoral and intracellular barriers. In order to solve these problems, various strategies are being devel-

oped which allow cytotoxic proteins to effectively penetrate cells and to block their activity during blood circulation, but are then activated near the target cells/tissues [43].

5. Conclusions

Quinoin isolated from quinoa seeds is a type 1 RIP that exhibits ROS-mediated genotoxic activity in the zebrafish model. Although this model organism appears to respond to treatment with quinoin by activating cellular defense mechanisms that reduce damage, significant *in vitro* and *in vivo* genotoxicity remained at all times. Further studies are needed to elucidate the prolonged genotoxicity of quinoin by effective DNA damage-response pathways, and to clarify the genotoxic mechanism of action by quinoin. The use of morpholinos to knockdown DNA damage response genes in zebrafish treated with quinoin could provide important insights in this area.

The acute genotoxic activity of quinoin opens up new possibilities for the use of this enzyme as a therapeutic tool in the development of new anticancer drugs.

However, non-specific cellular uptake of RIPs could potentially cause collateral toxicity [44]. As recently suggested by Asrorov *et al.* [45], these cytotoxic proteins could be tied to nanoparticles and to antibodies to improve the efficiency of tumor-targeted delivery.

Availability of Data and Materials

The data presented in this study are available on request from the corresponding author.

Author Contributions

All authors contributed to the study conception and design. Genotoxicity test were performed by FM, MC and RS. Quinoin purification and enzymatic assays were performed by SR and NL. The first draft of the manuscript was written by FM and LR, and all authors commented on previous versions of the manuscript. ADM and LR contributed with conceptualization, project administration, resources, funding acquisition, supervision, writing - review and editing. All authors read and approved the final manuscript and participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity. All authors contributed to editorial changes in the manuscript.

Ethics Approval and Consent to Participate

All used protocols were in accordance with the local regulation and international guidelines for the use and care of laboratory animals and approved by the Italian Ministry of Health (Permit Number: 529/2019-PR).

Acknowledgment

The authors would like to express their gratitude to Dr. Maria della Corte, Dr. Donatella Paciolla and Dr. Angela Barretta for their contribution in maintenance and treatment of zebrafish.

Funding

This work was supported by the research project PRR.AP026.014 - PNRR - IR0000009 - Title: '*Potentiating the Italian Capacity for Structural Biology Services in Instruct-ERIC*'. Acronym: ITACA.SB. Announcement: 'MUR D.D. 0003264 del 28/12/2021'. In addition, this work was supported by the University of Campania 'Luigi Vanvitelli' and University Federico II.

Conflict of Interest

The authors declare no conflict of interest. Given their role as Guest Editor Members, Filomena Mottola and Lucia Rocco had no involvement in the peer review of this article and had no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to Gheyath K. Nasrallah.

References

- [1] Prakash S, Gaiani C, Bhandari BR. Chapter 1 - Plant-based food as a sustainable source of food for the future. In Prakash S, Bhandari BR, Gaiani C (eds.) *Engineering Plant-Based Food Systems* (pp. 1–12). Academic Press: Cambridge, England. 2023.
- [2] Rietjens IMCM, Eisenbrand G. Chapter 1 - Natural toxicants in plant-based foods, including herbs and spices and herbal food supplements, and accompanying risks. In Knowles ME, Anelich LE, Boobis AR, Popping B. (eds.) *Present Knowledge in Food Safety* (pp. 2–25). Academic Press: Cambridge, England. 2023.
- [3] Salisbury FB, Ross CW. *Plant Physiology*. 4th edn. Wadsworth Publishing Company: Belmont, CA. 1992.
- [4] Popova A, Mihaylova D. Antinutrients in Plant-based Foods: A Review. *The Open Biotechnology Journal*. 2019; 13: 68–76.
- [5] Ram S, Narwal S, Gupta OP, Pandey V, Singh GP. 4 - Antinutritional factors and bioavailability: approaches, challenges, and opportunities. In Gupta OP, Pandey V, Narwal S, Sharma P, Ram S, Singh GP (eds.) *Wheat and Barley Grain Biofortification* (pp. 101–128). Woodhead Publishing: Sawston, England. 2020.
- [6] Stirpe F. Ribosome-inactivating proteins: from toxins to useful proteins. *Toxicon: Official Journal of the International Society on Toxinology*. 2013; 67: 12–16.
- [7] Stirpe F, Battelli MG. Ribosome-inactivating proteins: progress and problems. *Cellular and Molecular Life Sciences: CMLS*. 2006; 63: 1850–1866.
- [8] de Virgilio M, Lombardi A, Caliandro R, Fabbrini MS. Ribosome-inactivating proteins: from plant defense to tumor attack. *Toxins*. 2010; 2: 2699–2737.
- [9] Pastan I, Hassan R, FitzGerald DJ, Kreitman RJ. Immunotoxin treatment of cancer. *Annual Review of Medicine*. 2007; 58: 221–237.
- [10] Schlaak L, Weise C, Kuropka B, Weng A. Sapovaccarin-S1 and -S2, Two Type I RIP Isoforms from the Seeds of *Saponaria vaccaria* L. *Toxins*. 2022; 14: 449.
- [11] Polito L, Bortolotti M, Pedrazzi M, Mercatelli D, Battelli MG, Bolognesi A. Apoptosis and necroptosis induced by stenodactylin in neuroblastoma cells can be completely prevented through caspase inhibition plus catalase or necrostatin-1. *Phy-*

- tomedicine: International Journal of Phytotherapy and Phytopharmacology. 2016; 23: 32–41.
- [12] Bortolotti M, Maiello S, Ferreras JM, Iglesias R, Polito L, Bolognesi A. Kirkiin: A New Toxic Type 2 Ribosome-Inactivating Protein from the Caudex of *Adenia kirkii*. *Toxins*. 2021; 13: 81.
- [13] Landi N, Ruocco MR, Ragucci S, Aliotta F, Nasso R, Pedone PV, *et al.* Quinoa as source of type 1 ribosome inactivating proteins: A novel knowledge for a revision of its consumption. *Food Chemistry*. 2021; 342: 128337.
- [14] Bazile D, Jacobsen SE, Verniau A. The Global Expansion of Quinoa: Trends and Limits. *Frontiers in Plant Science*. 2016; 7: 622.
- [15] Angeli V, Miguel Silva P, Crispim Massuela D, Khan MW, Hamar A, Khajehei F, *et al.* Quinoa (*Chenopodium quinoa* Willd.): An Overview of the Potentials of the “Golden Grain” and Socio-Economic and Environmental Aspects of Its Cultivation and Marketization. *Foods (Basel, Switzerland)*. 2020; 9: 216.
- [16] Satheesh N, Fanta SW. Review on structural, nutritional and anti-nutritional composition of Teff (*Eragrostis tef*) in comparison with Quinoa (*Chenopodium quinoa* Willd.). *Cogent Food & Agriculture*. 2018; 4: 1546942.
- [17] Filho AMM, Pirozi MR, Borges JTDS, Pinheiro Sant’Ana HM, Chaves JBP, Coimbra JSDR. Quinoa: Nutritional, functional, and antinutritional aspects. *Critical Reviews in Food Science and Nutrition*. 2017; 57: 1618–1630.
- [18] El Hazzam K, Hafsa J, Sobeh M, Mhada M, Taourirte M, El Kacimi K, *et al.* An Insight into Saponins from Quinoa (*Chenopodium quinoa* Willd.): A Review. *Molecules (Basel, Switzerland)*. 2020; 25: 1059.
- [19] Rotondo R, Ragucci S, Castaldo S, Oliva MA, Landi N, Pedone PV, *et al.* Cytotoxicity Effect of Quinoin, Type 1 Ribosome-Inactivating Protein from Quinoa Seeds, on Glioblastoma Cells. *Toxins*. 2021; 13: 684.
- [20] Narayanan S, Surendranath K, Bora N, Surolia A, Karande AA. Ribosome inactivating proteins and apoptosis. *FEBS Letters*. 2005; 579: 1324–1331.
- [21] Canedo A, Rocha TL. Zebrafish (*Danio rerio*) using as model for genotoxicity and DNA repair assessments: Historical review, current status and trends. *The Science of the Total Environment*. 2021; 762: 144084.
- [22] Horzmann KA, Freeman JL. Making Waves: New Developments in Toxicology With the Zebrafish. *Toxicological Sciences: an Official Journal of the Society of Toxicology*. 2018; 163: 5–12.
- [23] Bhaskar ASB, Deb U, Kumar O, Lakshmana Rao PV. Abrin induced oxidative stress mediated DNA damage in human leukemic cells and its reversal by N-acetylcysteine. *Toxicology in Vitro: an International Journal Published in Association with BIBRA*. 2008; 22: 1902–1908.
- [24] Di Maro A, Chambery A, Carafa V, Costantini S, Colonna G, Parente A. Structural characterization and comparative modeling of PD-Ls 1-3, type 1 ribosome-inactivating proteins from summer leaves of *Phytolacca dioica* L. *Biochimie*. 2009; 91: 352–363.
- [25] Poerio E, Di Gennaro S, Di Maro A, Farisei F, Ferranti P, Parente A. Primary structure and reactive site of a novel wheat proteinase inhibitor of subtilisin and chymotrypsin. *Biological Chemistry*. 2003; 384: 295–304.
- [26] European Parliament. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the Protection of Animals Used for Scientific Purposes. *Official Journal of the European Union*. 2010; 276: 1–47.
- [27] Aleström P, D’Angelo L, Midtlyng PJ, Schorderet DF, Schulte-Merker S, Sohm F, *et al.* Zebrafish: Housing and husbandry recommendations. *Laboratory Animals*. 2020; 54: 213–224.
- [28] Mottola F, Iovine C, Santonastaso M, Carfora V, Pacifico S, Rocco L. Evaluation of Zebrafish DNA Integrity after Individual and Combined Exposure to TiO₂ Nanoparticles and Lincomycin. *Toxics*. 2022; 10: 132.
- [29] Santonastaso M, Mottola F, Iovine C, Cesaroni F, Colacurci N, Rocco L. In Vitro Effects of Titanium Dioxide Nanoparticles (TiO₂NPs) on Cadmium Chloride (CdCl₂) Genotoxicity in Human Sperm Cells. *Nanomaterials (Basel, Switzerland)*. 2020; 10: 1118.
- [30] Wong JH, Bao H, Ng TB, Chan HHL, Ng CCW, Man GCW, *et al.* New ribosome-inactivating proteins and other proteins with protein synthesis-inhibiting activities. *Applied Microbiology and Biotechnology*. 2020; 104: 4211–4226.
- [31] Wu W, Sun R. Toxicological studies on plant proteins: a review. *Journal of Applied Toxicology: JAT*. 2012; 32: 377–386.
- [32] Barbieri L, Polito L, Bolognesi A, Ciani M, Pelosi E, Farini V, *et al.* Ribosome-inactivating proteins in edible plants and purification and characterization of a new ribosome-inactivating protein from *Cucurbita moschata*. *Biochimica et Biophysica Acta*. 2006; 1760: 783–792.
- [33] Tang Y, Tsao R. Phytochemicals in quinoa and amaranth grains and their antioxidant, anti-inflammatory, and potential health beneficial effects: a review. *Molecular Nutrition & Food Research*. 2017; 61: 1600767.
- [34] Ballegaard ASR, Larsen JM, Rasmussen PH, Untersmayr E, Pilegaard K, Bøgh KL. Quinoa (*Chenopodium quinoa* Willd.) Seeds Increase Intestinal Protein Uptake. *Molecular Nutrition & Food Research*. 2021; 65: e2100102.
- [35] Melini V, Melini F. Functional Components and Anti-Nutritional Factors in Gluten-Free Grains: A Focus on Quinoa Seeds. *Foods (Basel, Switzerland)*. 2021; 10: 351.
- [36] Brigotti M, Alfieri R, Sestili P, Bonelli M, Petronini PG, Guidarelli A, *et al.* Damage to nuclear DNA induced by Shiga toxin 1 and ricin in human endothelial cells. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*. 2002; 16: 365–372.
- [37] Majtnerová P, Roušar T. An overview of apoptosis assays detecting DNA fragmentation. *Molecular Biology Reports*. 2018; 45: 1469–1478.
- [38] Atienzar FA, Jha AN. The random amplified polymorphic DNA (RAPD) assay and related techniques applied to genotoxicity and carcinogenesis studies: a critical review. *Mutation Research*. 2006; 613: 76–102.
- [39] Matic I. The major contribution of the DNA damage-triggered reactive oxygen species production to cell death: implications for antimicrobial and cancer therapy. *Current Genetics*. 2018; 64: 567–569.
- [40] Shih SF, Wu YH, Hung CH, Yang HY, Lin JY. Abrin triggers cell death by inactivating a thiol-specific antioxidant protein. *The Journal of Biological Chemistry*. 2001; 276: 21870–21877.
- [41] Rocco L, Izzo A, Zito G, Peluso C, Stingo V. Genotoxicity in Zebrafish (*Danio rerio*) Exposed to two Pharmacological Products from an Impacted Italian River. *Journal of Environmental and Analytical Toxicology*. 2011; 1: 1–7.
- [42] Liu W, Li PJ, Qi XM, Zhou QX, Zheng L, Sun TH, *et al.* DNA changes in barley (*Hordeum vulgare*) seedlings induced by cadmium pollution using RAPD analysis. *Chemosphere*. 2005; 61: 158–167.
- [43] Chen Y, Zhang M, Min KA, Wang H, Shin MC, Li F, *et al.* Improved Protein Toxin Delivery Based on ATTEMPTS Systems. *Current Drug Targets*. 2018; 19: 380–392.
- [44] Schwarze SR, Ho A, Vocero-Akbani A, Dowdy SF. In vivo protein transduction: delivery of a biologically active protein into the mouse. *Science (New York, N.Y.)*. 1999; 285: 1569–1572.
- [45] Asrorov AM, Gu Z, Min KA, Shin MC, Huang Y. Advances on Tumor-Targeting Delivery of Cytotoxic Proteins. *ACS Pharmacology & Translational Science*. 2019; 3: 107–118.