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Quinoa as source of type 1 ribosome inactivating proteins: A novel knowledge for a revision of its consumption

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ABSTRACT

This study investigates on the presence of toxic proteins in quinoa seeds. To this aim, a plethora of biochemical approaches were adopted for the purification and characterization of quinoin, a type 1 ribosome-inactivating protein (RIP) contained in quinoa seeds. We determined its melting temperature (68.2 ± 0.6 °C) and thermostability (loss of activity after 10-min incubation at 70 °C). Considering that quinoa seeds are used as a food, we found that quinoin is cytotoxic against BJ-5ta (human fibroblasts) and HaCaT (human keratinocytes) in a dose- and time-dependent manner. Moreover, in an *in vitro* digestive pepsin-trypsin treatment, 30% of quinoin is resistant to enzymatic cleavage. This toxin was found in seeds (0.23 mg/g of seeds) and in sprouted seeds obtained after 24-h (0.12 mg/g of sprout) and 48-h (0.09 mg/g of sprout). We suggest a thermal treatment of quinoa seeds before consumption in order to inactivate the toxin, particularly in sprouts, generally consumed raw.

1. Introduction

Chenopodium quinoa Willd. is an herbaceous annual plant, which has become in recent years a primary crop for its edible seeds. In particular, the growing popularity of quinoa seeds is due to the rich content of proteins, dietary fibers, B vitamins, and dietary minerals as well as for being a gluten-free food (Ceyhun Sezgin & Sanlier, 2019; FAO, 2011). In addition, quinoa seeds are known as functional food considering the benefit action on several human diseases (e.g. diabetes, dyslipidaemia, obesity and celiac disease) (Navruz-Varli & Sanlier, 2016). Consequently, in the last decades, the production of quinoa seeds and its derivatives (such as bread, pasta, sponge cakes or biscuits) had a continuous positive trend (Angeli et al., 2020).

On the other hand, quinoa seeds are not the elixir (panacea) or the 'golden grain' for human nutrition because of the high content of saponins, conferring a bitter taste and toxicity when present at high concentration; indeed, the elimination of saponins is strongly recommended. In addition, quinoa seeds are rich of phytic acid, tannins, oxalates and trypsin inhibitors, known as anti-nutritional factors (Filho, Pirozi, Borges, Pinheiro Sant'Ana, Chaves, & Coimbra, 2017). Hence, several methods (heat treatment, extrusion, roasting, or mechanical abrasion) are used to remove the excess of saponins and some anti-nutritional factors (El Hazzam et al., 2020).

Furthermore, *C. quinoa* belongs to Caryophyllales (Ceyhun Sezgin et al., 2019), a plant order known as a source of ribosome inactivating proteins (RIPs), toxins essentially produced by plants (Di Maro, Citores, Russo, Iglesias, & Ferreras, 2014; Stirpe & Gilabert-Oriol, 2017).

RIPs are enzymes (EC: 3.2.2.22) that damage ribosomes in an irreversible manner, leading to the arrest of protein synthesis (Stirpe & Gilabert-Oriol, 2017). In particular, RIPs are rRNA *N*-glycosylase, which remove a specific adenine (A^{4324} in rat) of the Sarcin Ricin Loop (known as SRL) in the 28S ribosomal RNA, involved in the interaction of the ribosome with the eukaryotic elongation factor 2 (EF-2) or the prokaryotic elongation factor G (EF-G), thus blocking translocation during the protein synthesis (Shi, Khade, Sanbonmatsu, & Joseph, 2012). The damage of the protein synthesis machinery is related to RIPs cytotoxicity, in turn triggering apoptosis pathway (Zeng, Zheng, Lu, Wang, Jiang, & Sha, 2015). Furthermore, it has been reported that some RIPs possess additional enzymatic activities on different substrates, including polynucleotide:adenosine glycosylase [PNAG (Barbieri, Valbonesi, Bonora, Gorini, Bolognesi, & Stirpe, 1997)], phosphatase activity on lipids, as well as DNase (Aceto et al., 2005), chitinase and

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superoxide dismutase activities (Stirpe & Gilabert-Oriol, 2017). From a structural point of view, RIPs are mainly classified into two groups, based on the presence or absence of a quaternary structure (Stirpe & Gilabert-Oriol, 2017). Indeed, type 1 RIPs are single-chain proteins with enzymatic action, whereas type 2 RIPs have an enzymatically active A chain linked to a B chain with lectinic properties. Another small number of RIPs has been grouped into type 3 RIPs, including some peculiar toxins in which the A chain is attached to peptidic segments that are then removed for activation (Zhu, Zhou, Ji, & Chen, 2018).

Type 2 RIPs are proteins endowed with higher toxicity due to the presence of a lectinic B chain bound to carbohydrate structures on the cell surface, allowing and sometimes facilitating the internalization of the enzymatic A chain into the cells. On the other hand, type 1 RIPs, lacking the lectinic chain, cannot bind to cells, entering with difficulty and consequently displaying a lower toxicity than that of ricin and related toxins. RIPs identified in several plant tissues (Ferreras et al., 1993) have been isolated from many angiosperms but not in gymnosperms (Di Maro et al., 2014; Lapadula & Ayub, 2017). Their physiological role is unknown; however, they have been reported to protect against herbivores, insects and viruses (Zhu et al., 2018). Given their toxicity, the presence of RIPs was investigated in several edible plants from which they were purified and characterized (Stirpe & Gilabert-Oriol, 2017). In some cases, these edible plants are not eaten raw, while others, such as seeds, fruits, leaves or other plant organs, are often eaten raw (Wu & Sun, 2012).

The aim of the present investigation was to verify the presence of RIPs in Caryophyllales and suggest cooking of quinoa seeds before consumption, considering the toxicity of these proteins (Stirpe & Gilabert-Oriol, 2017). Furthermore, because sprouted quinoa seeds are also heated raw, we have verified whether quinoin, a novel RIP found in quinoa seeds, was cytotoxic against normal human cells, such as fibroblasts or keratinocytes.

Our results highlight that the toxicity of quinoa seeds is not only due to the presence of saponins and other anti-nutritional factors, but also to the presence of RIPs or related toxins (Stirpe & Gilabert-Oriol, 2017). These data could justify the adaptive and innate immune responses observed in some patients with celiac disease, although quinoa seeds are gluten-free (Zevallos, Ellis, Suligoj, Herencia, & Ciclitira, 2012), as well as the insurgence of allergy and intolerance in healthy people who consume quinoa for the first time (Aurich, Simon, & Treudler, 2019).

2. Materials and methods

2.1. Materials

Chemicals for chromatography and Sodium Dodecyl Sulphate – PolyAcrylamide Gel Electrophoresis (SDS-PAGE) were obtained as previously reported (Di Maro, Chambery, Daniele, Casoria, & Parente, 2007; Landi et al., 2017). Medium 199 and other chemicals were from Sigma Chemical Co., St. Louis, MO, U.S.A. Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin G, streptomycin, trypsin were purchased from Lonza.

The following buffers have been used: buffer A: 5 mM Na-phosphate, pH 7.2, containing 0.14 M NaCl; buffer B: 10 mM Na-acetate, pH 4.0 and buffer C: 5 mM Na-phosphate pH 7.2.

2.2. Plant Material

Seeds of white quinoa (*Chenopodium quinoa* Willd) from Peru were purchased at local markets. In order to obtain sprouted quinoa, seeds were washed several times in tap water, drained off, left to soak for the night, drain off tap water, re-washed and then sprouted in the dark for 24- and 48-h at 25 °C, covered with a cloth moistened every 8-h.

2.3. Cell cultures

The cell line HaCaT, human immortalized keratinocyte cell line (Granato et al., 2017) was grown in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/mL penicillin G and 100 μg/mL streptomycin in humidified incubator at 37 °C under 5% CO_2 atmosphere. The cell line BJ-5ta, human skin fibroblasts immortalized with the human telomerase reverse transcriptase (Albano et al., 2013)] was cultured in a 4:1 mixture of DMEM and Medium 199 supplemented with 4 mM L-glutamine, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 10% FBS, 100 IU/mL penicillin G, 100 mg/mL and streptomycin in humidified incubator at 37 °C under 5% CO_2 atmosphere.

All cells were split and seeded every 3 days and used during their exponential phase of growth. Cell treatments were usually carried out after 24-h from plating.

2.4. Preparation of extracts and chromatographic steps for type 1 RIP purification

Seeds (100 g) were extracted by grinding with a Waring Blender (Waring Products; Torrington, USA) in buffer A (10 mL per g). After overnight stirring at 4 °C, extracts were strained through Miracloth filter paper (Merk Life Science; Milano, Italy) and centrifuged at 15,000 *g* for 45 min.

The pH of the crude extract was adjusted to pH 4.0 with glacial acetic acid, stirred at 4 °C for 1 h, and then centrifuged at 15,000 *g* at 4 °C for 1 h. The supernatant was loaded onto a column (i.d. 5 × 15 cm) containing Streamline™ SP (GE Healthcare; Milano, Italy) equilibrated in buffer B at 3 mL/min flow-rate. After sample loading, the resin was washed in buffer B (~0.7 L) and, then in buffer C (~0.9 L) , until the absorbance at 280 nm was below 0.01. Bound basic proteins were eluted with 1 M NaCl in buffer C, monitoring the absorbance of the eluate at 280 nm. The pool of basic proteins was concentrated in an Amicon cell concentrator (10 kDa-MWCO). The resulting protein sample (15 mL) was loaded onto a column (i.d. 3 × 120 cm) containing Sephacryl S-100 (GE Healthcare; 1–100 kDa separation range) equilibrated and eluted with 0.3 M NaCl in buffer C (flow rate 25 mL/h). Fractions (5 mL) with elution time corresponding to a molecular weight of ~30 kDa were pooled and dialysed against buffer C (4 °C) for 24-h. The dialyzed sample was further purified on a CM-Sepharose column (i.d. 1.4x25 cm), equilibrated in buffer C and eluted with a NaCl gradient up to 0.17 M (buffer C, 500 mL, buffer C containing 0.17 M NaCl, 500 mL; total volume 1 L). Single eluted fractions were assayed for depurination activity on hsDNA substrate. Purified enzyme was pooled, dialyzed against water, freeze-dried and stored at −20 °C until use.

2.5. Extraction of basic protein from sprouted quinoa

Sprouted quinoa (50 g) was subjected to the same procedure used for seeds until Streamline SP chromatographic step, by grinding with a Waring Blender in buffer A (5 mL per g), see below. Finally, the pool of basic proteins obtained after Streamline SP was concentrated, separated by gel-filtration on Sephadex 16/60 column (GE Healthcare; 1–100 kDa separation range; flow rate of 1 mL/min) by using AKTA Purifier 10 Fast protein liquid chromatography (FPLC) and characterised by densitometric analysis after SDS-PAGE separation.

2.6. Analytical procedures

All general methodologies used for analytical characterization of quinoin such as SDS-PAGE and determination of protein concentration by colorimetric assay (BCA assay), were carried out as previously described (Di Maro et al., 2007, 1999).

2.7. Enzymatic assays

The depurination assay (rRNA *N*-glycosylase assay) was conducted as previously described (Iglesias, Citores, Ragucci, Russo, Di Maro, & Ferreras, 2016). Briefly, rabbit reticulocytes lysates (40 μL), were incubated with 3.0 μg of protein at 37 °C for 1-h. After treatment, the RNA was subjected to phenolization, treated with 1 M aniline acetate (pH 4.5) and precipitated with ethanol. The RNAs were subjected to electrophoresis at 15 mA for 2 h in a 7 M urea/5% (w/v) polyacrylamide gel and stained with ethidium bromide.

Polynucleotide:adenosine glycosidase activity on salmon sperm DNA (adenine release) was measured according to a previously reported method (Di Maro et al., 2007).

2.8. DNA cleavage experiments

Endonuclease activity assays were performed at different conditions (in absence or presence of 5 mM Mg^{2+}, 0.25 mM Zn^{2+} or 25 mM EDTA) using pUC18 plasmid as substrate. Each reaction mixture (10 μL) was composed by 10 mM Tris•Cl, containing 50 mM NaCl and 50 mM KCl at pH 7.8, RIP (3.0 μg or 0.1 μg), substrate (200 ng) and all suitable chemicals to cover the different reaction conditions indicated above (Aceto et al., 2005). Samples were then incubated for 1 h at 37 °C, subsequently loaded (100 ng) on 0.8% (w:v) agarose gel in 40 mM Tris-acetate, 1.0 mM EDTA, pH 8.2 (TAE 1x) and finally analysed by ethidium bromide staining (0.5 μg/mL). *Eco*RI linearization was achieved by incubating 250 ng of pCR2.1 with 1.5 units of *Eco*RI enzyme according to manufacturer instructions (Sigma-Aldrich).

2.9. Circular dichroism and thermal stability determination

Far-UV CD spectrum was obtained at 25 °C on a Jasco J-815 dichrograph [Jasco Europe, Cremella (LC) Italy]. Far UV spectrum measurements were performed with a protein concentration of 0.3 mg/mL (10.3 μM) in 10 mM Na-phosphate, pH 7.2, using a 0.1 cm path-length quartz cuvette. DichroWeb (on-line analysis for protein Circular Dichroism spectra; http://dichroweb.cryst.bbk.ac.uk/html/home.shtml) was used to estimate the percentages of secondary structural elements (Whitmore & Wallace, 2008).

Protein (~0.10 mg/mL) in 10 mM sodium phosphate, pH 7.2 was subjected to heat-induced denaturation. Signals were monitored at 278 nm on a UV–VIS Cary 100 spectrometer [Agilent Technologies Italia S.p.A., Cernusco sul Naviglio (MI) Italy], equipped with a Peltier temperature controller. The temperature was raised (1 °C increments) from 20 °C to 95 °C at a rate of 1 °C/min. The fraction unfolded was calculated from observed absorbance and plotted against temperature. The temperature midpoint of the unfolding curve was determined by data fitting to the Boltzmann model using Prism 5 (GraphPad Software Inc., San Diego, CA, USA).

2.10. Protein thermal stability

The effect of temperature on enzymatic activity of quinoin was checked by incubating the purified enzyme in buffer C at increasing temperature, from 20 °C to 80 °C (Lam & Ng, 2001). After 10-min incubation at each temperature (20, 30, 40, 50, 60, 70 or 80 °C), aliquots (3.0 μg) were collected, kept on ice for 10 min and assayed with polynucleotide:adenosine glycosidase assay (see above).

2.11. Digestibility of proteins in vitro

The resistance of quinoin to gastro-intestinal (GI) digestion was assessed by using an *in vitro* pepsin-trypsin (P-T) enzyme system as previously reported (Wang et al., 2010) with some modifications. For the analysis, 100 μg of quinoin were dissolved in 0.1 M HCl (100 μL) in a 1.5 mL plastic tube. The ratio protease/substrate (E:S) was fixed at 1:100 for digestion. The digestion was conducted at 37 °C for 2-h. For SDS-PAGE analysis, 20-μL aliquots were withdrawn from reaction tube after 0, 30, 60 and 120-min incubation and put into 1.5 mL plastic tubes. To immediately inactivate pepsin, 0.1 M NaOH (20 μL) was added to each aliquot and then mixed with sample buffer (40 μL) before SDS-PAGE. Moreover, to verify also the effect of trypsin, after pepsin hydrolysis the mixture was subjected to trypsin digestion (E:S = 1:100) after pepsin inactivation by adding ammonium carbonate to a final concentration of 50 mM (final volume 400 μL), and incubating for additional 2 h at 37 °C. Aliquots (3 μg) were collected at each hour, and hydrolysis was terminated by boiling samples for 10 min, lyophilizing and then mixing with sample buffer (200 μL) before SDS-PAGE.

2.12. ATP assay

The ATP assay was used to detect cell proliferation, essentially as previously described (Avagliano et al., 2019). Briefly, BJ-5ta or HaCaT cells were seeded in 96-well microliter plates at 10×10^4 cells/well and let to grow for 24-h. Then, the cells were untreated or treated with different concentration of quinoin (0.0001 μM; 0.001 μM; 0.01 μM; 0.1 μM; 1 μM; 10 μM) for 48- and 72-h. After each incubation time, the ATP assay was performed using a CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega, Madison, WI, USA), according to the manufacturer's protocol.

2.13. Statistical analysis

The statistical analysis for polynucleotide:adenosine glycosidase was achieved by One-way ANOVA and post hoc Tukey's test (confidence range 95%) using Prism 5 (GraphPad Software Inc., San Diego, CA, USA). For ATP assay, the statistical significance of differences among groups was evaluated using student's *t*-test (Kaleida Graph v. 4.0, Reading, PA, USA). The significance was accepted at level of $p < 0.05$.

3. Results and discussion

3.1. Purification of Chenopodium quinoa type 1 ribosome inactivating protein

Crude extracts from seeds showed the ability to release adenines on salmon sperm DNA (PNAG activity). In order to verify whether this activity was due to the presence of type 1 RIPs, a protocol for extraction of basic proteins was followed (Di Maro et al., 2007, 1999). Total crude proteins extracted from seeds of Quinoa were subjected to acid precipitation to obtain the clarified protein extract. The supernatant fraction with PNAG activity was subjected to three chromatographic steps to obtain a homogeneous protein preparation: (i) preliminary cation exchange chromatography to select basic proteins; (ii) gel filtration chromatography (fractionation range of globular proteins 1–100 kDa); and (iii) cation exchange chromatography (Fig. 1A) where a mayor protein peak with PNAG activity was detected (data not shown). When fractions 205–215 were analysed by SDS-PAGE, a single protein band with an electrophoretic migration of about 29-kDa appeared (Fig. 1B, gel 1 and 2). Therefore, these fractions, corresponding to the active elution peak, were pooled, dialysed against water and used for further experiments considering their homogeneity on SDS-PAGE (Fig. S1). The yield of purified protein, hereafter quinoin, is ~3.0 mg/100 g of seeds.

3.2. rRNA N-glycosylase activity and other enzymatic action of quinoin

To ascertain whether the PNAG activity was due to the rRNA N-glycosylase activity characteristic of RIPs, we have verified whether purified quinoin is able to release the β-fragment (Endo's fragment) when incubated with rabbit ribosomes. As shown in Fig. 2A, quinoin depurinated the rRNA from rabbit reticulocyte ribosomes, releasing the

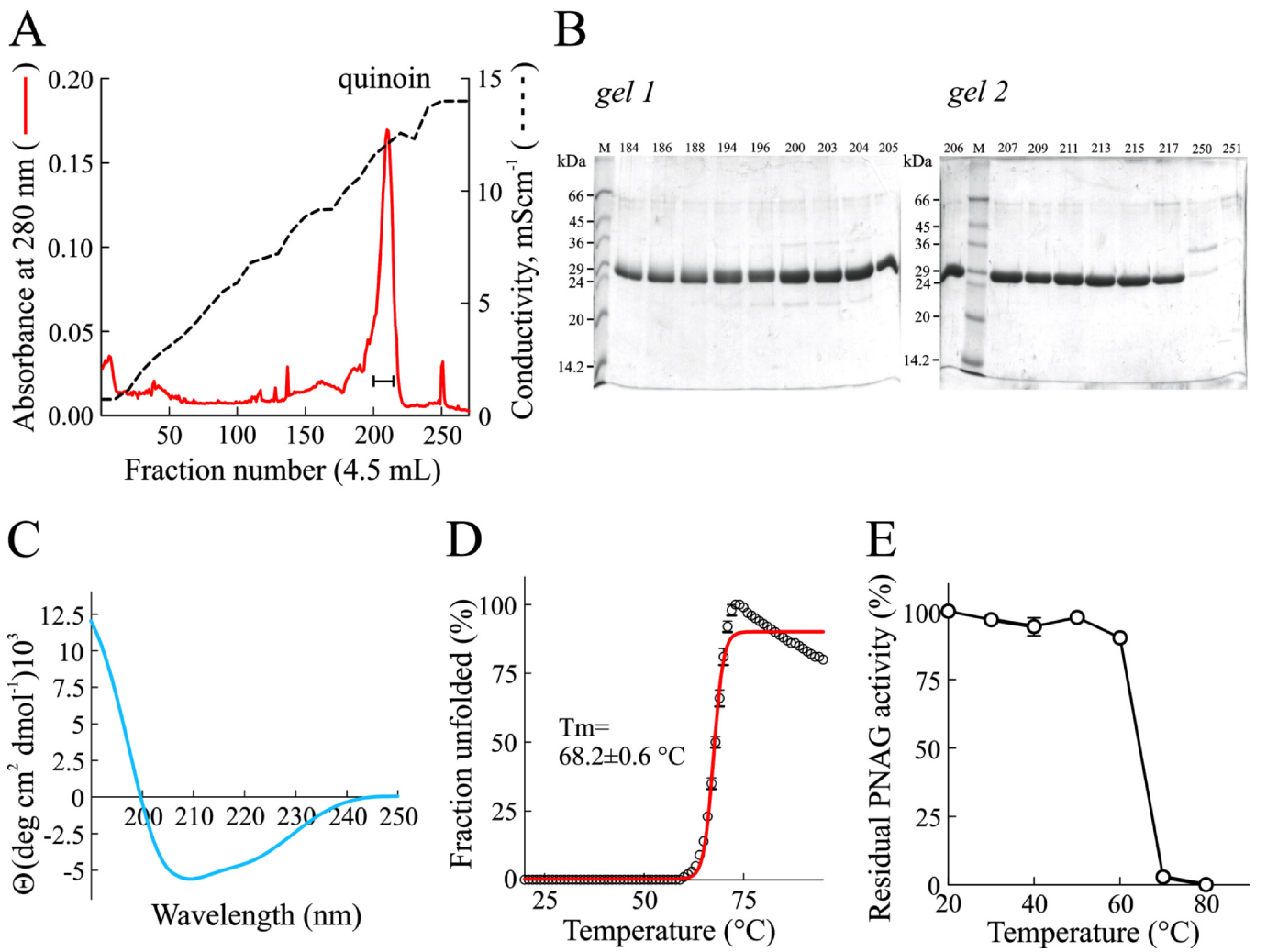

Fig. 1. (A), elution profile of the last purification step (exchange ion chromatography on CM-Sepharose column), showing one peak (quinoin) able to release β-fragment from ribosomes (see above). (B), SDS-PAGE (gel 1 and 2) analysis of single fractions (5.0 μg) obtained after cation exchange chromatography (Fig. 1A). M, molecular weight markers; SDS-PAGE with 2-mercaptoethanol was carried out in 15% polyacrylamide separating gel. (C) Far-UV CD spectrum of quinoin obtained as described in Materials and methods. (D) Thermal denaturation profile of quinoin (concentration; 0.1 mg mL^{-1}). Fraction unfolded at 278 nm is plotted as a function of temperature. The red line represents fit curve. (E) Effect of preincubation of quinoin at various temperatures for 10 min on its polynucleotide:adenosine glycosylase activity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

diagnostic RNA fragment, known as β-fragment upon acid aniline treatment as well as PD-L1, type 1 RIP isolated from leaves of *Phytolacca dioica* L. (Di Maro et al., 1999).

Furthermore, many RIPs exhibit a PNAG activity because they are able to release adenine from nucleic acids with different efficiency (Barbieri et al., 1997). As shown in Fig. 2B, quinoin displayed similar PNAG activity on salmon sperm DNA as well as PD-L1 and PD-S2 (type 1 RIP from seeds of *P. dioica*) (Iglesias et al., 2016). In particular, quinoin is less active than PD-S2 (~1.5-fold) and more active than PD-L1 (~1.3-fold).

Finally, another enzymatic activity associated to some RIPs is the endonuclease activity exerted on supercoiled plasmid DNA, thus producing relaxed or linear plasmids (Aceto et al., 2005). This capacity may expand the spectrum of RIPs actions as resistance to pathogenic microorganisms or viruses (Musidlak, Nawrot, & Goździcka-Józefiak, 2017). To this aim, we have tested the endonuclease activity of quinoin on the plasmid pUC18 DNA, and compared the results with the endonuclease activity of both PD-L1 and PD-S2. Quinoin, PD-L1 and PD-S2 promoted the conversion of supercoiled pUC18 DNA into the linear form (Fig. 2C). In particular, this action was evident for PD-L1 using 3.0 μg of enzyme (Fig. 2C, gel 1 and 2), whereas lower amounts of PD-S2 and quinoin (0.1 μg; Fig. 2C, gel 2) were used to obtain a similar effect. Indeed, the high basicity of these two proteins interferes with the electrophoretic migration of plasmid when 3.0 μg of protein were used in the assay, as emerging by the large smears at low mobility that appeared on agarose gel after enzyme incubation (Fig. 2C, gel 1). Moreover, the endonuclease activity of quinoin is not metal dependent, such as Mg^{2+} and Zn^{2+} (data not shown), as reported for other type 1 RIPs (Aceto et al., 2005; Iglesias et al., 2016).

3.3. Spectroscopic studies and thermal stability of quinoin

A study on the secondary structure of quinoin was performed by circular dichroism (CD) analysis. The far-UV circular dichroic spectrum of quinoin shows a protein profile with predominance of α-helical elements: two negative ellipticity signals at 222 and 208 nm and a positive ellipticity signal at 196 nm (Fig. 1C). Therefore, these data show that this type 1 RIP displays similar secondary structure content compared to other RIPs, which share a common three-dimensional fold (RIP fold) consisting of a C-terminal domain rich in α-helices and a β-stranded *N*-terminal domain (Chambery et al., 2007; Monzingo, Collins, Ernst, Irvin, & Robertus, 1993). Considering the near-UV CD spectrum

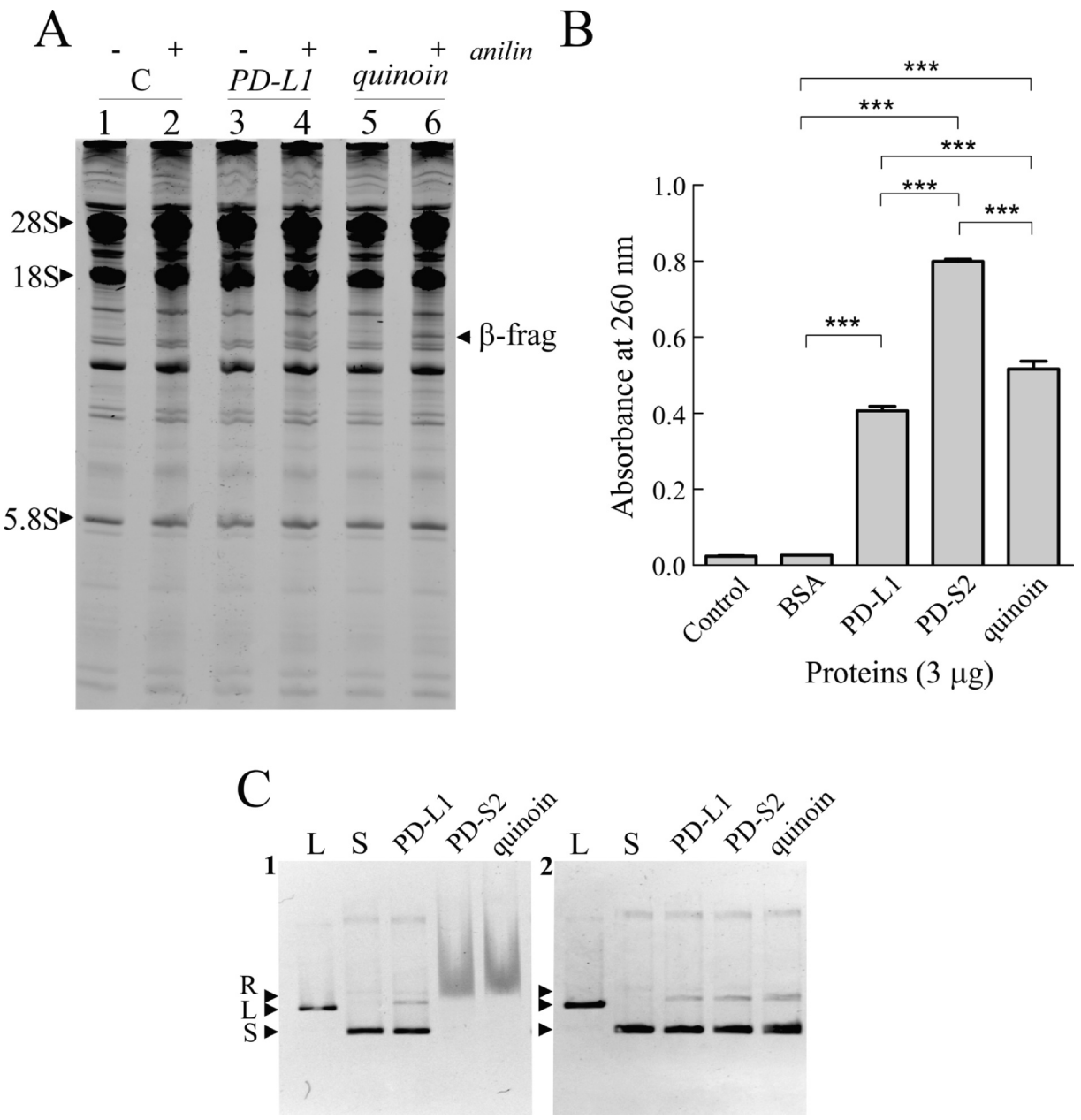

Fig. 2. (A) rRNA *N*-glycosylase activity assayed on rabbit ribosomes. Ribosomes were incubated with 3.0 μg of PD-L1, type 1 RIP from *P. dioica*, (lanes 3 and 4) as positive controls and 3.0 μg of quinoin (lanes 5 and 6). Following incubation, rRNA was extracted, treated with acid aniline and separated as reported in 'Material and methods' section. (+) and (-) indicate with and without aniline treatment. The 'β-frag' indicates the position of the Endo's fragment released by aniline treatment of rRNA from rabbit ribosomes. (B) Polynucleotide:adenosine glycosylase activity of BSA (negative controls) or type 1 RIPs (PD-L1, PD-S2 and quinoin) assayed on DNA. Proteins (3.0 μg) were assayed on salmon sperm DNA as described in Materials and methods. Data represent the mean of two duplicate experiments ± SE. Data were analysed by One-way ANOVA and *post hoc* Tukey's test (confidence range 95%). The asterisks indicate statistical significance between the indicated bar graph pairs (*** $p < 0.001$). (C) Endonuclease activity of PD-L1, PD-S2 and quinoin on pUC18 DNA. 200 ng/10 μL samples of plasmid DNA were incubated with 3.0 μg of PD-L1, PD-S2 and quinoin (gel 1) or 3.0 μg of PD-L1 and 0.1 μg of PD-S2 and quinoin (gel 2) as indicated in Materials and methods. L: pUC18 DNA was previously linearized using *Eco*RI. R, L, and S indicate relaxed, linear and supercoiled forms of pUC18, respectively.

(Fig. S2) quinoin displays a principal ellipticity signal in the range of 295–310 and 250–270 nm, regions where mainly the protein tryptophan and phenylalanine residues absorb, respectively (Whitmore et al., 2008).

Likewise, thermal denaturation curve of quinoin was obtained using UV-spectroscopy by measuring increment of absorbance at 278 nm, increasing the temperature. The melting temperatures (Tm) of quinoin was 68.2 ± 0.6 °C (Fig. 1D). Thermal unfolding curve at pH 7.2 shows that this RIP is a stable protein. In addition, quinoin displays an evident thermostability, considering that after 10 min of incubation up to 60 °C, 90% of residual PNAG activity is retrieved (Fig. 1E), while at 70 °C the enzymatic activity is lost, (~3% of residual PNAG activity).

3.4. Toxic effect of quinoin in non-tumour cell lines

When quinoa is used as a food, its seeds are usually cooked, although, in some cases, quinoa sprouts are consumed raw. Because it is known that raw quinoa seeds are toxic, we have evaluated the possible toxicity of quinoa sprouts on normal human cells, such as BJ-5ta and HaCaT cells, a human immortalized fibroblast cell line and human immortalized keratinocyte cell line, respectively.

BJ-5ta and HaCaT cell lines were incubated with vehicle alone or

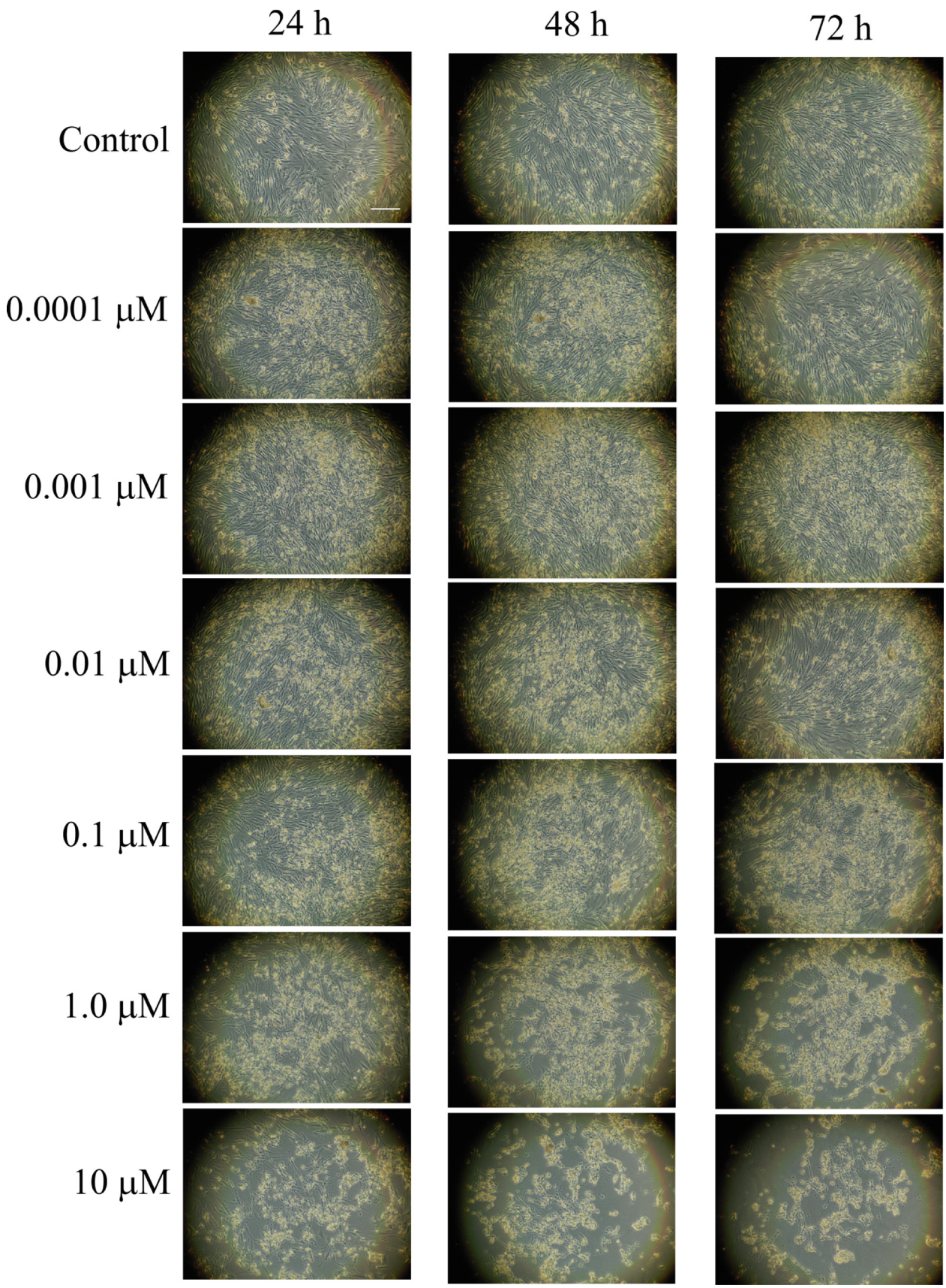

Fig. 3. Effect of quinoin on the morphology of BJ-5ta cells. BJ-5ta cells were treated with 0.0001 μM, 0.001 μM, 0.01 μM, 0.1 μM, 1.0 μM and 10 μM quinoin for 24-, 48- and 72-h. The control is represented by untreated cells. Scale bar 200 μm. Magnification ×10.

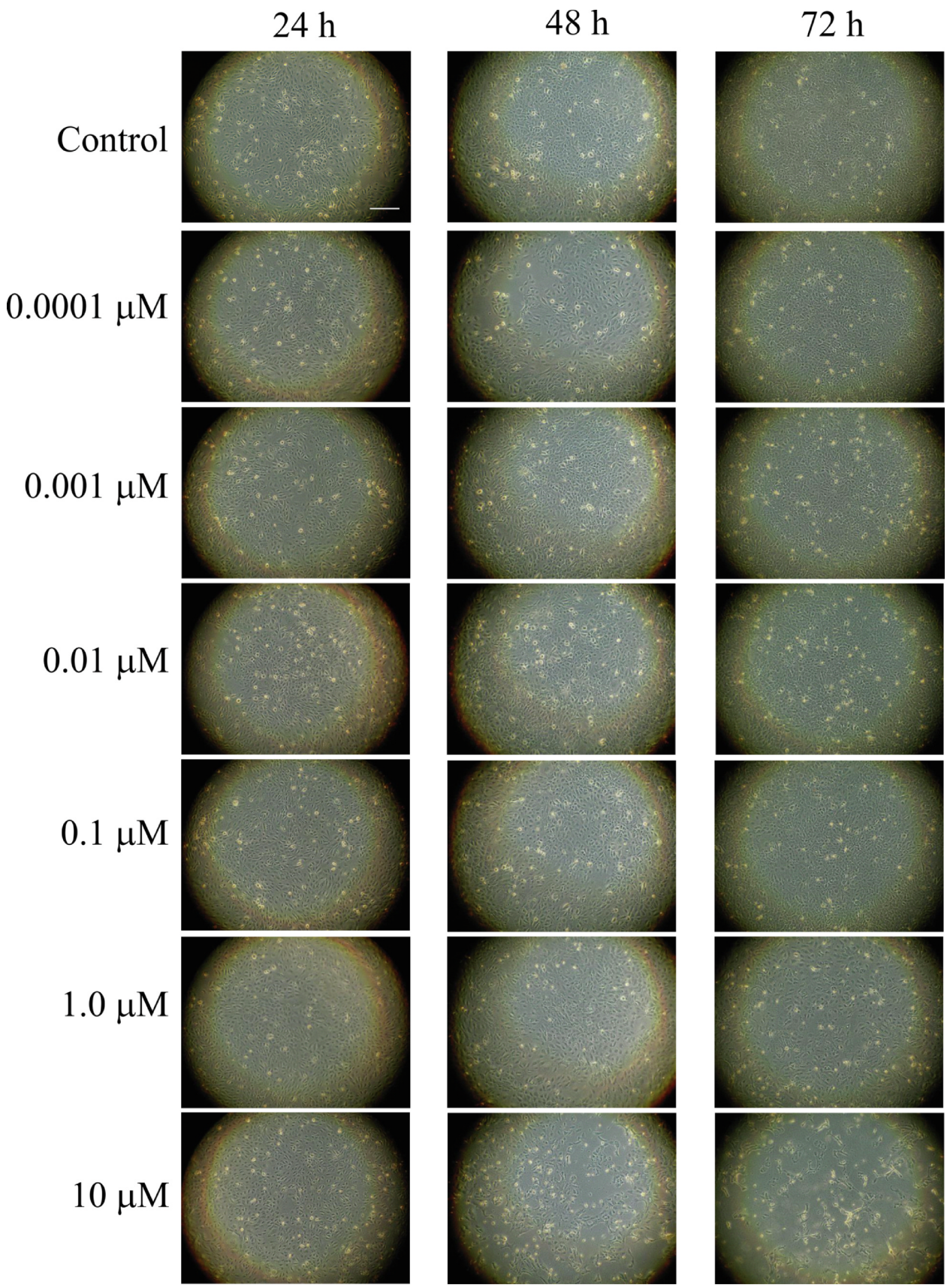

Fig. 4. Effect of quinoin on the morphology of HaCaT cells. HaCaT cells were treated with 0.0001 μM, 0.001 μM, 0.01 μM, 0.1 μM, 1 μM and 10 μM quinoin for 24-, 48- and 72-h. The control is represented by untreated cells. Scale bar 200 μm. Magnification ×10.

A

Plant material (100g)	sprouting time (days)	Raw protein extract (mg of protein/g of materials)	Basic proteins (mg of protein/g of materials)	Type 1 RIP (mg of protein/g of materials)
quinoa seeds	0	28.70±1.43	1.89±0.08	0.23±0.01
sprouted seeds	1	9.95±0.45	0.67±0.03	0.12±0.01
sprouted seeds	2	1.35±0.07	0.42±0.02	0.09±0.00

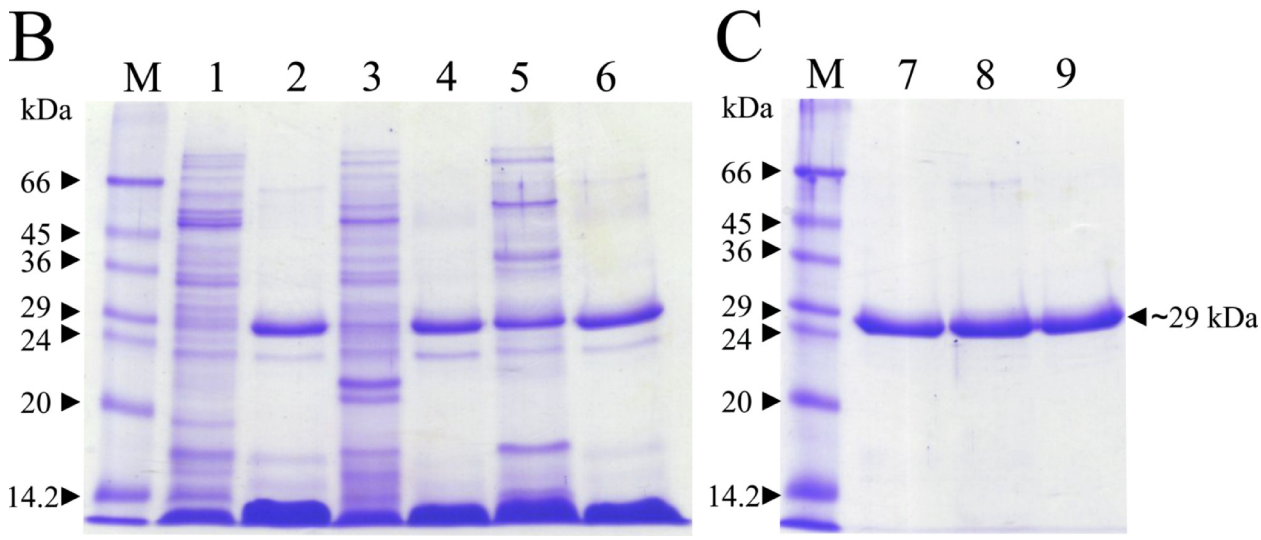

Fig. 5. Analytical purification of proteins from quinoa seeds and sprouted quinoa. (A) Raw soluble proteins, basic proteins and quinoin content from seeds or sprouted quinoa after 24- and 48-h reported as mg per gram of plant materials. (B) SDS-PAGE profile of raw soluble proteins and basic proteins from seeds (lanes 1 and 2) or sprouted seeds after 24-h (lanes 3 and 4) and 48-h (lanes 5 and 6). (C) Pool of basic proteins with an elution volume of 29-kDa obtained from quinoa seeds (lane 7) or sprouted quinoa after 24-h (lane 8) and 48-h (lane 9).

with increasing concentrations of quinoin (0.0001 μM, 0.001 μM, 0.01 μM, 0.1 μM, 1.0 μM, 10 μM) and then photographed after 24-, 48- or 72-h by light microscopy to evaluate morphological changes. As shown in Fig. 3 and Fig. 4, the quinoin treatment provoked cell morphological alterations such as cell shrinkage, nuclear condensation and a reduced cell number in a time- and dose-dependent manner. In particular, the effect of quinoin on cell morphology of BJ-5ta cells was already evident after 24-h of treatment with 10 μM quinoin, became more pronounced after 48- and 72-h treatment, and observed even in the presence of 1 μM quinoin (Fig. 3). HaCaT cells were less sensible to the effect of quinoin because the morphological alterations were essentially evident after 48- and 72-h of treatment with 10 μM of quinoin (Fig. 4).

In order to investigate on the toxic action of quinoin, the ATP assay was performed to evaluate its effect on BJ-5ta and HaCaT cell viability. Data shown in Fig. S3 clearly indicated that quinoin reduced the cell viability of both BJ-5ta and HaCaT cells, in a dose- and time dependent manner. Furthermore, in agreement with the morphological results, BJ-5ta cells were more sensible to the toxic effect of quinoin respect to HaCaT cells. Indeed, a significant reduction of BJ-5ta cell viability was already observed by treatment with 0.1 μM (Fig. 3, panel A) or 0.001 μM quinoin (Fig. 3, panel C) after 48- and 72-h, respectively.

The influence of quinoin on HaCaT cell viability was also in line with morphological observations. Indeed, the minimum dose of quinoin provoking a significant reduction of HaCaT cell viability was 1.0 μM, both after 48- and 72-h. Hence, the sensibility of normal fibroblasts and keratinocytes to toxic effect of quinoin lead us to suggest a very careful use of raw quinoa sprouts in diet.

3.5. Purification from sprouted quinoa

The consumption of sprouted seeds is alternative to quinoa seeds, considering that the sprouting process of quinoa seeds leads to an increase in nutritional values and antioxidant activity and a decrease in levels of phytate, protease inhibitors and other antinutritional factors (Alvarez-Jubete, Arendt, & Gallagher, 2009; Lim, Park, & Yoon, 2020; Zhang, Xing, Sun, Zhou, Ren, & Qin, 2020). However, the finding of toxic quinoin in seeds suggests an investigation on the presence of this toxin during seed germination to form sprouted quinoa, monitoring its amount at increasing germination times. In particular, seeds were placed in a germinator at 25 °C and collected after 24- and 48-h. As control, a parallel purification was performed using seeds before they were incubated in the germinator. After collecting samples, the amount of raw protein extract, basic proteins and the proteins contained in the 29-kDa peak after gel-filtration were quantified, using the BCA assay (Fig. 5, panel A) and subjected to SDS-PAGE analysis (Fig. 5, panel B). In particular, the analysis revealed a reduction in the amount of basic proteins and quinoin (band 29-kDa after gel-filtration step) at increasing germination time. On the other hand, quinoin amount in relative terms (percentage) increase after sprouting time (1.20% and

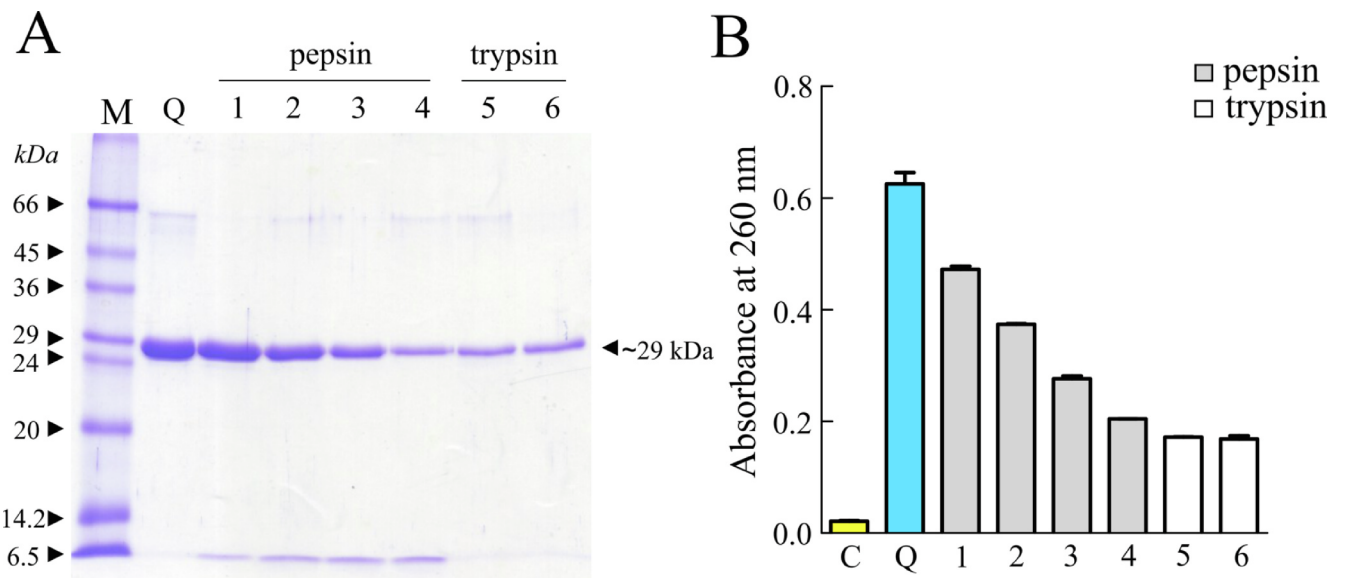

Fig. 6. (A) SDS-PAGE profile of quinoin subjected to *in vitro* protein digestibility. Pepsin treatment at 0, 30, 60 and 120 min (lanes 1, 2, 3 and 4, respectively) and subsequent trypsin treatment after 60 or 120 min of pre-treatment with pepsin (lanes 5 and 6, respectively). Q, purified quinoin; M, molecular weight markers. SDS-PAGE was carried out in 15% polyacrylamide separating gel under reducing conditions. (B) Residual polynucleotide:adenosine glycosylase activity of quinoin subjected to *in vitro* protein digestibility. Pepsin treatment at 0, 30, 60 and 120 min (grey bars 1, 2, 3 and 4, respectively) and subsequent trypsin treatment after 60 or 120 min (white bars 5 and 6, respectively) of pre-treatment with pepsin. Q, purified quinoin. For more details, see Material and methods. For statistical analysis, see supplementary materials (Fig. S5).

6.70% at day 1 and 2, respectively, with respect to raw protein extract (0.80%). Indeed, the amount of quinoin in sprouted seeds at 24- and 48-h decreases of 1.9-fold (0.12 mg/g) and 2.6-fold (0.09 mg/g), respectively, respect to the type 1 RIP retrieved in quinoa seeds (0.23 mg/g), Fig. 5**,** panel A.

Finally, the activity of 29-kDa pooled peak (3.0 μg) obtained after gel-filtration from seeds (lane 7, Fig. 5C), sprouted quinoa at 24-h (lane 8, Fig. 5C) and 48-h (lane 9, Fig. 5C) detected by PNAG assay on salmon sperm DNA compared to purified quinoin (3.0 μg) remains constant (Fig. S4).

Hence, the data show that, despite its decrease, the toxin is still present as active enzyme in sprouted quinoa.

3.6. Digestibility of quinoin in vitro

Considering the cytotoxicity of quinoin on both BJ-5ta and HaCaT cell lines, its possible ingestion when quinoa seeds are not subjected to properly thermal treatment or the consumption of sprouted seeds as raw food, an *in vitro* study on digestibility of quinoin was carried out. Indeed, a rapid degradation of protein toxins could attenuate or eliminate toxic effects due to the destabilization of acid conditions of the stomach that aids their hydrolysis by pepsin or subsequent hydrolysis by trypsin in the small intestine (Swaisgood & Catignani, 1991). Thus, we have treated quinoin with the common digestive pepsin-trypsin system (Wang et al., 2010) at different times and the effect was evaluated by SDS-PAGE.

Pepsin-trypsin digestion of quinoin displays that part of the toxin is hydrolysed by pepsin in acid conditions (Fig. 6A). Indeed, after 120 min of proteolytic treatment with pepsin, we observe a decrease of about 67% of the toxin, while no difference in the SDS-PAGE profile was found when samples were pre-treated with pepsin and then subjected to trypsin digestion at 60 and 120 min. Moreover, to verify the enzymatic capability of quinoin (residual band of 29-kDa) in the digested mixtures obtained at different times, the residual PNAG activity on salmon sperm DNA was assayed. The results display a decrease of enzymatic activity in the samples digested with pepsin with respect to undigested quinoin (Fig. 6B, Q bar and grey bars). On the other hand, samples pre-treated with pepsin and then subjected to trypsin treatment do not display a further reduction of PNAG activity, retaining a residual activity of 30% (white bars Fig. 6B).

The resistance to pepsin digestibility confirms previous studies on ebulin f and SELfd, type 2 RIPs found in fruits of dwarf elder (*Sambucus ebulus* L.) (Carrillo, Cordoba-Diaz, Cordoba-Diaz, Girbés, & Jiménez, 2017; Jimenez, Tejero, Cabrero, Cordoba-Diaz, & Girbes, 2013).

4. Conclusions

The protocol applied for extraction of basic proteins from *C. quinoa* seeds allowed us to purify at homogeneity a novel RIP, named quinoin. This enzyme has the properties of type 1 RIPs: (i) single-chain protein with a molecular weight of ~ 29 kDa; and (ii) enzymatic rRNA *N*-glycosylase activity on rabbit ribosomes releasing β-fragment after aniline treatment. In addition, quinoin displays other enzymatic features common to type 1 RIPs, such as PNAG activity on salmon DNA and endonuclease activity on supercoiled plasmid DNA that is not metal dependent.

Subsequently, we have determined the Tm of quinoin and the effect of heat treatment. Quinoin is a stable protein (Tm = 68.2 °C) and quite resistant to thermal treatment, preserving ~95% of its activity until 60 °C, and losing 97% of its enzymatic activity after 10 min at 70 °C. Furthermore, considering the ingestion of this toxin when quinoa seeds are not subjected to properly thermal treatment or the consumption of sprouted seeds as raw food, a study on digestibility of quinoin *in vitro* was carried out. Our results indicate that quinoin, in an *in vitro* system, is partially resistant to pepsin-trypsin treatment, because 30% of this toxin is resistant to treatment and retains the enzymatic activity. Thus, these data could be useful for tuning a heat treatment able to inactivate this toxin, especially in sprouted quinoa that usually is eaten raw. Furthermore, our data show that the germination led to a time-dependent decrease of quinoin amount, although this decrease does not impair its enzymatic activity in sprouted quinoa.

Hence, the toxic effect exerted by quinoin in both normal fibroblast and keratinocytes indicates that the edible use of raw quinoa sprouts is not recommended. Furthermore, the high quinoin sensibility exhibited by fibroblasts, an ubiquitously cell type regulating tissue homeostasis, prompted us to speculate that the use of raw quinoa sprouts could

provoke side-toxic effect (Arcucci et al., 2011; Hematti, 2012; Ruocco et al., 2018). However, to better validate our hypothesis on the toxic effects deriving from the edible use of quinoa, studies *in vivo* should be performed, although it should be emphasized that the toxicity of quinoin, type 1 RIP, is less strong than type 2 RIPs (Stirpe & Gilabert-Oriol, 2017).

Overall, the consumption of quinoa seeds may result in harmful effects not only for the presence of saponins and other anti-nutritional factors, generally eliminated with several methods (heat treatment, extrusion, roasting, or mechanical abrasion), but also for the presence of a toxin, such as quinoin, capable of blocking protein synthesis.

Considering the sensibility of normal fibroblasts and keratinocytes to the toxic effect of quinoin, we suggest a very careful use of seeds and sprouted quinoa in diet, particularly referring to the sprouts, generally consumed raw. Hence, a thermal treatment is preferred before consumption, capable of inactivating the toxin.

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CRediT authorship contribution statement

Nicola Landi: Investigation. **Maria Rosaria Ruocco:** Writing - review & editing, Funding acquisition. **Sara Ragucci:** Investigation. **Federica Aliotta:** Investigation. **Rosarita Nasso:** Investigation. **Paolo V. Pedone:** Writing - review & editing, Funding acquisition. **Antimo Di Maro:** Conceptualization, Writing - original draft, Methodology, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.128337.

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