Antioxidant activity and protective effect of the outer scales hydroalcoholic extract of *Allium cepa* L. var. Tropea on toxicity damage induced by Cadmium in Caco-2 cells

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Abstract

Allium cepa L., the common onion, is widely used in the human diet and it has been recognized as an important reservoir of valuable phytonutrients and micronutrients such as polyphenols and flavonoids. The present study investigated the *in vitro* protective effects against Cadmium (Cd) of the hydroalcoholic extract from the outer scales of Tropea red onion (*A. cepa* L. var. Tropea) in human Caco-2 colon adenocarcinoma cell line and its *in vitro* antioxidant effects. The DPPH assay revealed a good radical scavenging activity of the extract with an IC_{50} value of 14.76 ± 0.44 µg/mL; a significantly higher antioxidant activity was observed when the β-carotene-linoleate bleaching assay was applied, with IC_{50} values of 5.41 ± 0.02 and 17.78 ± 0.12 µg/mL after 30 and 60 minutes of incubation, respectively. In addition, the extract inhibited NO release in a concentration-dependent manner, with an IC_{50} value of 235.7 ± 10.78 µg/mL. Exposure of Cd treated human Caco-2 cells to the Tropea red onion extract resulted in a higher cytoprotection with a significant reduction of cells damage induced by Cd. and with an increase in viability. The effect of Tropea red onion evaluated with the MTT assay and LDH test, was time-dependent and more evident after 24h of treatment. The evaluation of cell viability on Caco-2 adenocarcinoma cell line of the heavy metal was carried out with concentrations ranging from 0.01 to 250 µM at 24 and 72h. *A. cepa* extract at increasing concentrations 25, 50 and 100 µg/mL caused an increase in cell viability with a dose and time dependent trend. The treatment of the cells with the mixture of *A. cepa* extract and CdCl2 at 24h

showed a significant cytoprotection at concentrations equal to 50 µg/mL + 25 µM and 100 µg/mL + 25 µM while at 72h at a concentration of 25 µg/mL + 25 µM. We observe with MTT and LDH assays that treatment of cells with *A. cepa* at 24h was able to significantly prevent CdCl2-induced cytotoxicity alone.

Keywords: Tropea red onion; radical scavenging; lipid peroxidation; NO; human adenocarcinoma; cadmium; quercetin; phenolics

1. Introduction

It is well known that antioxidants play important roles in animal health by inactivating harmful free radicals produced through normal cellular activity and from various stressors (Fatma M El-Demerdash, Mokhtar I Yousef, Fatma S Kedwany, Hoda H Baghdadi., 2004 Cadmium-induced changes in lipid peroxidation, blood hematology, biochemical parameters and semen quality of male rats: Protective role of vitamin E and β-carotene *Food and Chemical Toxicology 42 1563–1571*) and contribute to reduce the risk for developing chronic diseases such as coronary heart disease (CHD), cancer, diabetes and Alzheimer's disease (Ravi Kant Upadhyay, 2016 Garlic: A potential source of pharmaceuticals and pesticides: A review *International Journal of Green Pharmacy, Jan-Mar (Suppl) 10 (1)*).

Cadmium (Cd) is one of the most concerned environmental pollutant possessing high toxicity for humans, animals and plants (Rukhsanda Aziz M T Rafiq, Jie Yang, Di Liu, Lingli Lu, Zhenli He, M K Daud , Tingqiang Li, Xiaoe Yang 2014 Impact Assessment of Cadmium Toxicity and Its Bioavailability in Human Cell Lines (Caco-2 and HL-7702) *BioMed Research International Volume 2014, Article*). It is a non-essential transition metal produced from various agriculture and industrial sources which can accumulate in living organisms with a long half-life of about 25-30 years. Exposure to Cd is considered one of the most severe health risk. It has been demonstrated that Cd is able to stimulate free radical production, resulting in oxidative deterioration of lipids, proteins and DNA thus leading to the development of serious pathological conditions in humans and animals (V Lobo, A Patil, A Phatak, N Chandra 2010 Free radicals, antioxidants and functional foods: impact on human health.*Pharmacognosy Reviews, July-December, Vol 4 Issue*).

It is known that once absorbed, Cd binds to the thiol groups of cysteine residues of low molecular weight proteins, the metallothioneins, and accumulates in the kidneys, liver and reproductive organs (Fatma M El-Demerdash, Mokhtar I Yousef, Fatma S Kedwany, Hoda H Baghdadi., 2004 Cadmium-induced changes in lipid peroxidation, blood hematology, biochemical parameters and semen quality of male rats: Protective role of vitamin E and β-carotene *Food and Chemical Toxicology 42 (2004)*

1563–1571). High concentrations of Cd are also deposited in the pancreas and salivary glands. In addition to peripheral organs, the central nervous system is also subjected to Cd toxicity (Lafuente A, Esquifino AI (1999) Cadmium effects on hypothalamic activity and pituitary hormone secretion in the male. Toxicol Lett 110:209–218). Cd can enter the brain parenchyma and neurons, causing neurological alterations in humans and animal models (Jomova K, Valko M (2011) Advances in metal-induced oxidative stress and human disease. Toxicology 283:65–87)Furthermore, Cd affects cell proliferation, differentiation, apoptosis and other cellular activities, interfere with antioxidant defence mechanisms and inhibition of oxidative DNA repair systems; it also stimulates the production of reactive oxygen species which may act as signalling molecules in the induction of gene expression and apoptosis (Michael Waisberg, Pius Joseph, Beverley Hale, Detmar Beyersmann Molecular and cellular mechanisms of cadmium carcinogenesis *Toxicology* 192(2003) 95–117.; G Bertin, D Averbeck Cadmium: cellular effects, modifications of biomolecules, modulation of DNA repair and genotoxic consequences (a review) *Biochimie* 88 (2006) 1549–1559). In recent years, much evidence supports the implication of Cd as potential risk cofactors in Alzheimer's disease (AD)(Bocharova OV, Breydo L, Salnikov VV, Baskakov IV (2005) Copper(II) inhibits in vitro conversion of prion protein into amyloid fibrils. Biochemistry 44:6776–6787; Bush A (2000) Metals and neuroscience. Curr Opin Chem Biol 4:184–191; Ricchelli F, Drago D, Filippi B, Tognon G, Zatta P (2005) Aluminum-triggered structural modifications and aggregation of beta-amyloids. Cell Mol Life Sci 62:1724–1733) favouring the formation of amyloid beta peptides different-sized aggregates and increasing their cytotoxicity (G. Notarachille, F. Arnesano, V. Calò, D. Meleleo, Heavy metals toxicity: effect of cadmium ions on amyloid beta protein 1-42. Possible implications for Alzheimer's disease, Biometals. 27 (2) (2014) 371–388; D. Meleleo, C. Sblano, M.M. Storelli, R. Mallamaci. Evidence of cadmium and mercury involvement in the Aβ42 aggregation process, Biophysical Chemistry. 266 (2020) 106453. https://doi.org/10.1016/j.bpc.2020.106453). Several chelating agents and antagonists are established to reduce Cd toxicity (Pari L, Murugavel P, Sitasawad SL, Kumar KS. Cytoprotective and antioxidant role of diallyl tetrasulfide on cadmium induced renal injury: an

in vivo and in vitro study *Life Sci*. 80 (2007) 650–658) and several authors have shown that antioxidants should be one of the important components of an effective treatment of Cd poisoning (Casalino E., Calzaretti G, Sblano C, Landriscina C. Molecular inhibitory mechanisms of antioxidant enzymes in rat liver and kidney by cadmium *Toxicology* 179 (2002) 37–50 ;Fatma M El-Demerdash, Mokhtar I Yousef, Fatma S Kedwany, Hoda H Baghdadi., 2004 Cadmium-induced changes in lipid peroxidation, blood hematology, biochemical parameters and semen quality of male rats: Protective role of vitamin E and β-carotene *Food and Chemical Toxicology 42 1563–1571*). However, the detailed mechanisms by which toxic metals such as Cd produce their effects are still largely unknown. *Allium cepa* L. is widely used in the human diet and it has been recognized as an important reservoir of valuable phytonutrients (Yanyan Zhou, Cong Li,Bang Feng,Bang Chen,Lihua Jin,Yehua Shen 2020. UPLC-ESI-MS/MS based identification and antioxidant, antibacterial, cytotoxic activities of aqueous extracts from storey onion (*Allium cepa* L. var. *proliferum* Regel) *Food Research International*). It is a rich source of micronutrients such as polyphenols and flavonoids for which there may be differences in composition, concentration and beneficial activities depending on the cultivar and variety. *A. cepa* L. var. Tropea, commonly known as Tropea red onion, is a unique variety cultivated in the Calabria region (southern Italy). This cultivar has recently received a lot of interest due to its phytochemical and biological characterization. Its bulbs feature a characteristic red external tunic and white internal scales and have appreciated organoleptic properties such as sweet taste, tenderness and crispness.

Our previous study demonstrated that the hyroalcoholic extract obtained from the dry outer scale leaves of Tropea red onion has inhibitory activity *in vitro* against pancreatic lipase and could be exploited as a promising anti-obesity agent (Marrelli, M., Russo, C., Statti, G., Argentieri, M. P., Meleleo, D., Mallamaci, R., Pinarosa Avato & Conforti, F. (2022). Phytochemical and biological characterization of dry outer scales extract from Tropea red onion (*Allium cepa* L. var. Tropea): a promising inhibitor of pancreatic lipase. *Phytomedicine Plus*, 100235).

As a continuation of our previous investigation, the present study aims to be a further contribution to the overall knowledge of the biopotential of the hydroalcoholic extract from the dry outer scales of Tropea red onion. As far as we know, this is the first example of simultaneous administration of Tropea red onion extract and $CdCl_2$, in order to highlight its protective effect against the toxicity damage induced by Cd. Cadmium is a widespread toxic metal which accumulates in the environment and produces a broad spectrum of toxicological effects to various organs in humans and animals. Following oral exposure or ingestion, Cd first reaches and damages the intestinal epithelium. Moreover, there are some experimental evidences indicating that oxidative stress and reactive oxygen species generated in presence of Cd are involved in its toxic effects. In this Based on the above evidences, in our study we have employed human Caco-2 adenocarcinoma cell line, known as the "golden standard" of *in vitro* intestinal cell models (Hubatsch et al.2007), to investigate the protective effect of Tropea red onion extract against Cd. The cytotoxic effect was evaluated *in vitro* by MTT and LDH cytotoxicity assays following two different strategies of treatment: direct and simultaneous with *$CdCl_2$* for 24 and 72 hrs. Finally, the *in vitro* antioxidant effects of the extract were also evaluated to disclose its potential antioxidative protection.

2. Materials and methods

2.1 Chemicals

All solvents used for extraction and chemical analyses were of analytical grade and were purchased from VWR International s.r.l. (Milan, Italy). 1,1-diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid, β-carotene, linoleic acid, propyl gallate, Tween 20, CdCl2, lipopolysaccharide (LPS) ?????,

High glucose (4.5 gL^{-1}) Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased PAN Biotech), L-glutamine, Trypsin-EDTA, 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Cadmium chloride (CdCl2), NADH were purchased from Sigma-Aldrich S.p.a. (Milan, Italy). Penicillin and streptomycin were purchased from LONZA Bioscience,

Human Caco-2 adenocarcinoma cells, RAW 264.7 murine macrophages, …, were purchased from … .

2.2 Plant material

Tropea red onion outer dry scales (*A. cepa* L. var. Tropea) were obtained from onion bulbs collected in southern Italy (Calabria region) in September 2018 (voucher specimen CLU 25978 deposited at the Botanic Garden, University of Calabria). The hydroalcoholic extract was obtained by maceration with 70% aqueous EtOH solution as solvent as described in Marrelli et al. (2022) (Mariangela Marrelli, Concetta Russo, Giancarlo Statti, Maria Pia Argentieri, Daniela Meleleo, Rosanna Mallamaci, Pinarosa Avato, Filomena Conforti. Phytochemical and biological characterization of dry outer scales extract from Tropea red onion (*Allium cepa* L. var. Tropea)–A promising inhibitor of pancreatic lipase. Phytomedicine Plus 2 (2022) 100295).

2.3. *Compositional analysis of the extract*

The freshly prepared hydroalcoholic extract was analyzed for its content of phenolics. It was preliminarly checked by TLC eluted with EtOAc:HCO_2H:CH_3CO_2H:H_2O (100:11:11:27, v/v). Phosphomolybdic acid reagent (10% EtOH) or alternatively Natural Products-Polyethyleneglycol reagent (NP-PEG, Sigma) were used to visualize the extract components. After spraying, TLC plates were dried off at 110 °C on a Camag TLC Plate Heater 3 and spots observed under visible-253 nm or in UV-366 nm light. Components in the extract were quantified and identified by HPLC-DAD and HPLC-HRMS following the analytical procedures already reported in Marrelli et al. (2022).

2.4 Evaluation of the antioxidant activity

The antioxidant activity of the above extract was investigated through two *in vitro* assays: the DPPH (1,1-diphenyl-2-picrylhydrazyl) test and the β-carotene bleaching test. The free radical DPPH was utilized for the determination of the radical scavenging potency of the analyzed samples, as

previously described (Amodeo, V., Marrelli, M., Pontieri, V., Cassano, R., Trombino, S., Conforti, F., & Statti, G. (2019). *Chenopodium album* L. and *Sisymbrium officinale* (L.) Scop.: Phytochemical content and in vitro antioxidant and anti-inflammatory potential. *Plants*, *8*(11), 505). Test solutions of the extract at different concentrations (5-1000 µg/ml) were added to a 10^{-4}M MeOH solution of DPPH. Absorbance was measured at 517 nm after 30 minutes incubation in the dark using a Perkin Elmer Lambda 40 UV/VIS spectrophotometer. Assays were run in triplicate and ascorbic acid was used as a positive control.

The anti-lipid peroxidation activity of the extract was assessed by the β-carotene/linoleic acid system [Marrelli, M., Araniti, F., Statti, G., & Conforti, F. (2019). Metabolite profiling and biological properties of aerial parts from *Leopoldia comosa* (L.) Parl.: Antioxidant and anti-obesity potential. *South African Journal of Botany*, *120*, 104-111]. A chloroformic 0.5 mg/ml β-carotene solution (1 mL) was added to linoleic acid (0.02 mL) and Tween 20 (0.2 mL). The solvent was evaporated and 100 ml of water was added to the residue to obtain an emulsion; a total of 0.2 ml of each sample (at a concentration ranging from 0.025 to 100 µg/ml) was then added to 5 ml of the above mixture. Obtained solutions were placed in a water bath at 45 °C and absorbance was measured at 470 nm at initial time, and again after 30 and 60 minutes of reaction. Bleaching assays were run in triplicate and propyl gallate was used as a positive control. The antioxidant activity was measured in terms of successful prevention of β-carotene bleaching.

2.5 Inhibitory properties on nitric oxide production

The ability of *A. cepa* hydroalcoholic extract to inhibit nitric oxide (NO) production was evaluated on lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophages as previously reported (Marrelli, M., Argentieri, M. P., Avato, P., & Conforti, F. (2020). *Lobularia maritima* (L.) Desv. Aerial Parts Methanolic Extract: In Vitro Screening of Biological Activity. Plants, 9(1), 89). Cells were cultured at 37 °C under 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with fetal bovine serum (10%), L-glutamine (1%) and a solution 100 U/ml penicillin

(5%), and 100 mg/mL streptomycin (5%). To carry out the experiments, cells were removed from the flask by scraping and seeded onto 96 wells-microplates (100000 cells/well). The next day, medium was replaced with fresh DMEM containing 1 µg/mL lipopolysaccharide (LPS) and the *A. cepa* extract at different concentrations (25–1000 µg/mL in EtOH 70%, final ratio of solvent to medium 0.5% v/v). The presence of nitrite, a stable end product of NO oxidation, was verified in cell culture media 24 h later. 100 µL of Griess reagent were added to 100 µL of the culture supernatant and absorbance was measured at 550 nm. Both indomethacin and the known NO synthase inhibitor L-NAME were used as positive controls. The absence of cytotoxic effects of samples on treated cells was verified using the MTT test.

2.6 *Preparation of Cadmium solutions*

Cadmium was administered as the water soluble salt $CdCl_2$. A stock solution of $CdCl_2$ was prepared by dissolving under stirring 0.2283g of $CdCl_2$ powder, in 10 mL of bidistilled sterile water and subsequent filtration. Final concentration was 1×10^{-1} M. The CdCl2 solutions at concentration ranging from 0.01 to 250 µM were obtained from stock solution by scalar dilution. The solutions were stored at 4 °C until use.

2.7 Preparation of the extract solutions

The extract of Tropea red onion 5 mg/ml and sterilized by filtration through 0.22 µm micro filters (Sartorius, Germany) was used. Serial dilutions of extract were added in the culture medium to achieve the required final concentrations from 25, 50 to 100 µg/mL

2.8 Culture cells

Human Caco-2 adenocarcinoma cells were used in this study. The cells were cultured in 25 cm^2 flasks (Corning Inc., NY, USA) and maintained in high glucose (4.5 gL^{-1}) Dulbecco's modified Eagle

medium (DMEM), supplemented with 10% (*v*/v) fetal bovine serum (FBS, PAN Biotech), 4 mM L-glutamine (SIGMA), and 1% (v/v) antibiotic solution 100 U/ml penicillin (5%), and 100 mg/mL streptomycin (5%). The cells were maintained at 37°C in the incubator (Thermo Scientific Hera Cell 240i) with 5% CO_2 in air atmosphere and 95% relative humidity. After being 80% confluent, the cells were washed with a phosphate-buffered saline solution (PBS) to remove any unattached cells. The attached cells were harvested using 1 mL of 0.25% trypsin and 0.53 mM EDTA solution and then seeded at the density of (5000 cells/well) in 96-well plate and incubated for 24 h to allow attachment before treatment with $CdCl_2$ and the extract. Cell monolayers were washed with phosphate buffered saline-PBS to remove any unattached cells. The attached cells were harvested using a 1 mL 0.25% trypsin and 0.53 mM EDTA solution and then seeded at a density of 5000 cells/well in a 96-well plate and incubated for 24 h to allow attachment. In order to monitor the toxicity of $CdCl_2$, Tropea red onion extract and $CdCl_2$/Tropea red onion mixtures on Caco-2 cells, we prepared the following experimental sets:

- in the first experimental set, cells were exposed to increasing concentration of $CdCl_2$ (0.01, 0.05,0.25, 2.5, 25 and 250 µM), 6 wells/concentration group and incubated for 24 and 72 h. Only $CdCl_2$ was added to the culture media.
- in the second experimental set, cells were exposed to increasing concentration of Tropea red onion extract 25, 50 and 100 µgr/mL and incubated for 24 h and 72h. Only the extract was added to the culture media.
- in the third experimental set, cells were exposed to mixtures of $CdCl_2$ (25 µM)/Tropea red onion extract at scalar concentrations of 25, 50 and 100 µgr/ml. and incubated for 24 and 72 h.

Control groups consisted of Caco-2 cells in media which were processed identically and incubated simultaneously as the treated groups (D.Romero, M Gómez-Zapata, A Luna, A J García-Fernández Morphological characterisation of BGM (Buffalo Green Monkey) cell line exposed to low doses of cadmium chloride 2003 *Toxicol. In vitro 17, 293-299*).

2.9 Determination of cell viability

Cell viability was tested with MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide) method. The assay is based on the ability of living cells to convert dissolved MTT into insoluble formazan by mitochondrial dehydrogenases in viable cells. The amount of formazan produced is proportional to the number of living cells (S Amoroso, A Gioielli, M Cataldi, G Di Renzo, L Annunziato In the neuronal cell line SH-SY5Y, oxidative stress-induced free radical overproduction causes cell death without any participation of intracellular Ca(2+) increase *Biochimica et Biophysica Acta 1452 (1999)*). In brief, after 24 and 72 hrs, media containing various concentrations was removed and the cells incubated with 20 µl of MTT stock solution (5 mg/ml in PBS 1X) in 180 µl of medium. Mi sembrava spiegato meglio nella precedente versione: In brief, cells were seeded at the density of 5×10^4 in a 96-well plate and incubated for 24 h. Cells were exposed to increasing concentrations of $CdCl_2$ and Tropea red onion extract (6 wells/concentration group plus 1 control group) and incubated for 24 and 72 hrs. After this time, medium was removed and incubated with 20 µl of MTT stock solution (5 mg/ml in PBS 1X) in 180 µl of medium. Following additional 3 h incubation at 37°C in the dark, the medium was removed and 150 µl of DMSO was added to dissolve the formazan crystals and agitated for 5 min at room temperature. Finally, the absorbance was measured at 540nm using a multilabel microplate reader Victor 3 (PerkinElmer). All MTT assays were performed in triplicate. Cell viability is expressed as a percentage of the control group. Cell MTT response (% control) was calculated from the equation: *% control =Absorbance treatment/Absorbance control× 100%.* Data are the mean percentages of viable cells versus the respective controls. OK si può cambiare

2.10 Lactate Dehydrogenase (LDH) Release

Cytotoxicity was assessed by lactate dehydrogenase (LDH) leakage into the culture medium. Following exposure to the $CdCl_2$ and Tropea red onion extract, the culture medium was aspirated and centrifuged at 3000 rpm for 5 min to obtain a cell free supernatant. The LDH assay is based on the conversion of lactate to pyruvate in the presence of LDH with parallel reduction of NAD to

NADH. The change in the absorbance was recorded at 440 nm using a microplate spectrophotometer system (Bio-Rad-680, Bio-Rad, USA). Cell LDH release (% control) was calculated with the equation: *% control = (U LDH/mg cell protein) treatment / (U LDH/mg cell protein) control × 100%.* (Simon Kaja, Andrew J Payne, Yuliya Naumchuk, Peter Koulen 2018 Quantification of Lactate Dehydrogenase for Cell Viability Testing Using Cell Lines and Primary Cultured Astrocytes *Curr Protoc Toxicol.; 72: 2.26.1–2.26.10.*)

2.11 Statistical analysis

Data were expressed as mean ± SEM of three independent experiments. Normality of data was estimated using D'Agostino–Pearson's K2 test and homogeneity of variances was assessed using Levene's test. The fitting procedure was carried out using the GraphPad Prism 5 software (GraphPad Prism™ version 3.0) statistical software package. Differences with respect to the control group were statistically analyzed using One-way ANOVA followed by Dunnett's multiple comparison test (SigmaStat Software, SanRafael, CA, USA).

Potrebbe andare questa fusione dei due paragrafi? Noi (unical) abbiamo usato la versione 3 di Prism (come in articolo precedente). Poiché abbiamo paragonato sempre ai controlli in tutti i test fatti (sia Unical che UniBa), va bene per voi se uniformiamo, e mettiamo come post-hoc solo il Dunnett, che si presta non al confronto "all pairwise", ma "versus control"?
OK

3. Results and discussion

3.1. Phenolic composition of the hydroalcoholic extract

Compositional profile of the freshly prepared hydroalcoholic extract from the dry outer scales of Tropea red onion has been checked by HPLC-UV-DAD and HPLC-HRMS analyses. As expected (Marrelli et al. 2022), the extract was rich in phenolics. The chromatographic and spectrometric data allowed to confirm that the main specialized metabolites were quercetin derivatives with quercetin (86 μg mL^{-1}), spiraeoside (44 μg mL^{-1}) and quercetin-3,4′-*O*-diglucoside (14 μg mL^{-1}) as the three

dominant constituents. Quercetin-7,4′-diglucoside (2 µg mL^{-1}), alliumoside A (6 µg mL^{-1}) and isoquercitrin (6 µg mL^{-1}) were also present in the extract. As already described (Marrelli et al. 2022), this newly prepared extract for this investigation also contained the pigment cynidin-3-*O*-glucoside (1 µg mL^{-1}), likely responsible of the red color of the bulb scales.

3.2 Antioxidant activity

The DPPH free radical scavenging activities of the hydroalcoholic extract from Tropea red onion is described in Figure 1a. Extract showed a good radical scavenging activity with an IC_{50} value of 14.76 ± 0.44 µg/mL. In addition, it was found that the hydroalcoholic extract exhibited a high antioxidant activity also when the β-carotene-linoleate bleaching assay was applied, with IC_{50} values equal to 5.41 ± 0.02 and 17.78 ± 0.12 µg/mL after 30 and 60 minutes of incubation, respectively (Fig. 1b,c). Celano and coworkers (2021) recently assessed the antioxidant activity of the hydroalcoholic extract from the outer dry protective layers of *A. cepa* L. var. Tropea using ABTS scavenging capacity and ORAC assays (Celano, R., Docimo, T., Piccinelli, A. L., Gazzerro, P., Tucci, M., Di Sanzo, R., & Rastrelli, L. (2021). Onion Peel: Turning a Food Waste into a Resource. *Antioxidants*, 10(2), 304). Results were expressed as Trolox equivalent antioxidant capacity (TEAC) *per* g of extracts (µmol TE/g), and were equal to 7.82 ± 0.72 and 11.32 ± 1.40 µmol TE/g for ORAC and ABTS assay, respectively. The biological activity of Tropea red onion has been previously demonstrated also by Tedeschi and colleagues (Tedeschi, P., Bonetti, G., Maietti, A., & Brandolini, V. (2014). Random amplified polymorphic DNA (RAPD) fingerprint and antioxidants profile as markers for Tropea red onion (*Allium cepa* L.) authenticity. *Journal of Food Composition and Analysis*, 36(1-2), 98-103), who assessed the antioxidant capacity of water-soluble compounds from the bulb extract by photochemiluminescence method. Others authors reported the antioxidant capacity of the onion bulb extract using the ferric reducing antioxidant power (FRAP) assay and demonstrated the reduction of LDL oxidation and the protection of human erythrocytes from oxidative damage induced by HClO (Tedesco, I., Carbone, V., Spagnuolo, C., Minasi, P., & Russo, G. L. (2015). Identification and

quantification of flavonoids from two southern Italian cultivars of *Allium cepa* L., Tropea (Red Onion) and Montoro (Copper Onion), and their capacity to protect human erythrocytes from oxidative stress. *Journal of agricultural and food chemistry*, 63(21), 5229-5238.)

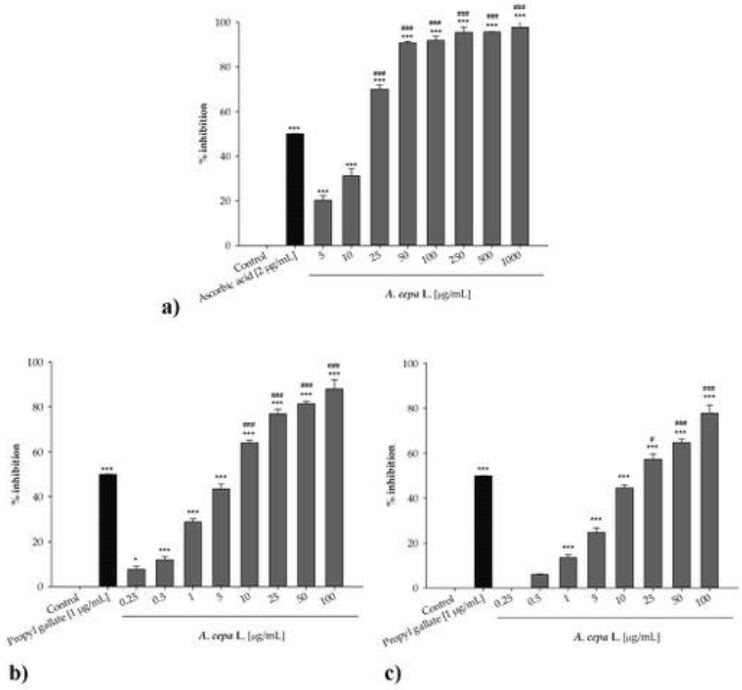


Figure 1. Free radical scavenging activity (a) and inhibition of lipid peroxidation after 30 and 60 minutes of incubation (b and c) of the Tropea red onion hydroalcoholic extract. Data are expressed as mean ± SEM (n=3). Ascorbic acid (2 μg/mL) and propyl gallate (1 μg/mL) were used as positive controls. Significant differences *versus* control: * $p < 0.05$, *** $p < 0.001$; significant differences *versus* positive control: $^{\#}$ $p < 0.05$, $^{\#\#\#}$ $p < 0.001$ (Dunnett's multiple comparison test).
Anche in questa figura bisogna sostituire Allium cepa con Tropea red onion extract?? non è mia

3.3 Inhibition of NO production

The inhibitory potential of the hydroalcoholic extract on NO production was verified *in vitro* in LPS-stimulated RAW 264.7 macrophages. The presence of nitrite, a stable oxidized product of nitric oxide, was verified in cell culture medium by means of the Griess reagent. At the highest tested concentration, 1000 μg/mL, the sample was able to induce 84.40 ± 1.07% inhibition of nitric oxide production ($p < 0.001$ compared to control, Dunnett's multiple comparison test), without showing

any cytotoxic effect on treated cells. The extract inhibited nitric oxide release in a concentration-dependent manner, with an IC_{50} value of 235.7 ± 10.78 µg/mL (Figure 2).

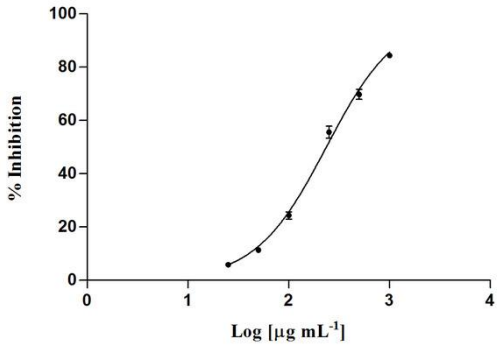
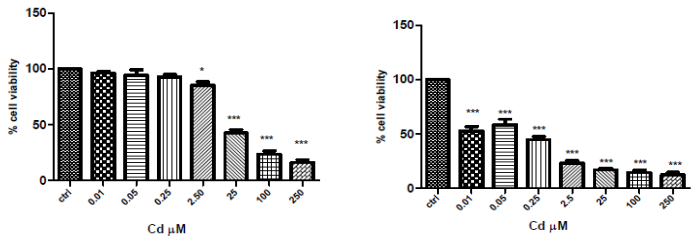


Figure 2. Concentration-dependent *in vitro* inhibitory activity on NO production of Tropea red onion outer scales hydroalcoholic extract.

Singh and co-workers (2009) evaluated the scavenging of nitric oxide radical of EtOAc fraction of red onion peel collected in India and extracted with Soxhlet apparatus. They demonstrated that, using the sodium nitroprusside method, the EtOAc fraction showed strong inhibitory effect on nitric oxide radical and values varied from 21.64 ± 1.76% to 93.92 ± 4.34% in a concentration-dependent manner (100–500 µg/ml) (Singh, B. N., Singh, B. R., Singh, R. L., Prakash, D., Singh, D. P., Sarma, B. K., Upadhyay G. & Singh, H. B. (2009). Polyphenolics from various extracts/fractions of red onion (Allium cepa) peel with potent antioxidant and antimutagenic activities. *Food and Chemical Toxicology*, *47*(6), 1161-1167). The onion peel was also investigated as regard the inhibition of NO production in the murine macrophage cell line RAW 264.7 cells. The results of this study showed that NO levels decrease in a dose-dependent manner when the cells were treated with hot water extract obtained from the *A. cepa* peel (Kang, B. K., Kim, K. B. W. R., Ahn, N. K., Choi, Y. U., Kim, M. J., Bark, S. W., Pak W.-M., Kim B.-R., Park J.-H., Bae N.-Y., & Ahn, D. H. (2015). Anti-inflammatory effect of onion (Allium cepa) peel hot water extract in vitro and in vivo. *KSBB Journal*, *30*(4), 148-154).

3.4 Cytotoxic activity

In order to determine the effect of Cd on Caco-2 cells, a MTT cell viability assay was carried out. Cells exposed to $CdCl_2$ from 0.01 to 250 µM exhibited decreasing percentages of cell viability with the increasing of $CdCl_2$ concentration, showing significant differences in cell viability compared with the control at 250 µM $CdCl_2$ concentration, after 24 h exposure. Cd cytotoxicity is more evident after 72 h' exposure. (Fig 3) The results obtained indicate that an exposure time of 24 h is sufficient to appreciate Cd toxic effect.



a) b)

Fig. 3. Effect of $CdCl_2$ on Caco-2 cell viability after 24 h (a) and 72 h (b) of exposure by MTT assay. Data are expressed as mean ± SEM (n=3). Significant differences *versus* control ***$P < 0.05$).

In questa figura non sono riportati asterischi, ma nella didascalia sì. Ho inserito asterischi

Potremmo uniformare, come di consueto, con $^{\#}p < 0.05$, $^{**}p < 0.01$, $^{\#\#\#}p < 0.001$? si ok

To evaluate the effect of Tropea red onion extract alone on Caco-2 cell viability, cytotoxicity experiments were performed by treating the cells with the extract alone at concentrations of 25, 50 and 100 µg/mL for 24 h and 72h incubation (Fig. 4). The results show a significant increase of the cells viability compared with the control after 24 h exposure in a dose dependent manner while after

72 h exposure a decrease from 50% to 80%.was observed at 100 µg/mL and 50 µg/mL, respectively?

NON so se intendo bene il concetto si ok

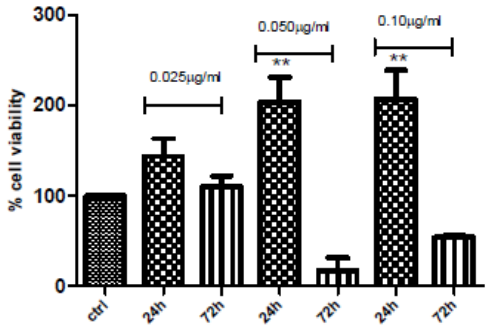
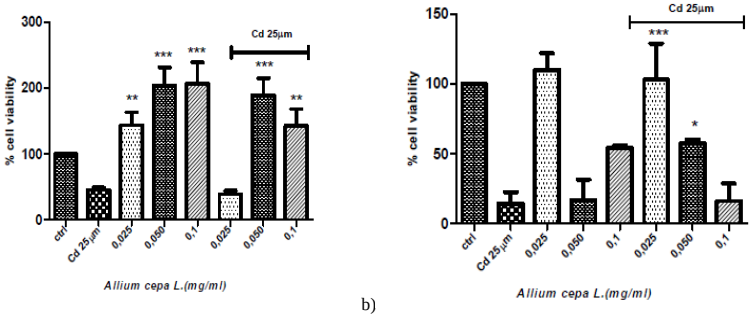


Fig. 4. Effect of Tropea red onion hydroalcoholic extract on Caco-2 cell viability after 24 h and 72 h of exposure by MTT assay. Data are expressed as mean ± SEM (n=3). Significant differences *versus* control $^{***}P < 0.05$).

Potremmo uniformare, come di consueto, con $^{\#}p < 0.05$, $^{**}p < 0.01$, $^{\#\#\#}p < 0.001$? si ok

By applying the same procedure described above (MTT assay), the effects of Caco-2 cells exposure to mixtures of $CdCl_2$/Tropea red onion extract at the concentrations described above are shown in Fig (5). Compared to the control, a remarkable cytoprotection was observed when mixtures of Tropea red onion extract and $CdCl_2$ were used at 50 µg/mL + 25 µM and 100 µg/mL + 25 µM for 24h incubation, and at 25 µg/mL + 25 µM for 72h incubation, respectively



a) b)

Fig. 5. Effects of simultaneous strategy treatment with Tropea red onion and $CdCl_2$ on Caco-2 cell viability after 24 h (a) and 72 h (b) of exposure by MTT assay. Data are expressed as mean ± SEM (n=3). Significant differences *versus* control $^{***}P < 0.05$) Potremmo uniformare, come di consueto, con $^{*}p < 0.05$, $^{**}p < 0.01$, $^{###}p < 0.001$?

NELLA FIGURA si può cambiare Allium cepa con Tropea red onion? Si

The results were further validated with the lactate dehydrogenase (LDH) assay by estimating the toxicity of the compound; this enzyme is rapidly released into the cell culture supernatant when the plasma membrane is damaged, a key feature of cells undergoing apoptosis, necrosis, and other forms of cellular damage (George Fotakis, John A. Timbrell, 2006 In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride *Toxicology Letters 160 171–177)* (Ana Flávia L Specian· Juliana M Serpeloni, Katiuska Tuttis, Diego L Ribeiro, Heloísa L Cilião, Eliana A Varanda, Miriam Sannomiya,Wilner Martinez-Lopez, Wagner Vilegas, Ilce M S Cólus 2016 LDH, proliferation curves and cell cycle analysis are the most suitable assays to identify and characterize new phytotherapeutic compounds *Cytotechnology* 68:2729–2744.

The results obtained showed that LDH release in Caco-2 cells treated with 25 µM $CdCl_2$ is reduced by adding mixtures of Tropea red onion extract and $CdCl_2$ (100 µg/mL + 25 µM after 24 hours); on the contrary, at the same conditions, the LDH released was less significant after 72 h. The protective

effects of Tropea red onion extract against $CdCl_2$ are described in Fig. 6

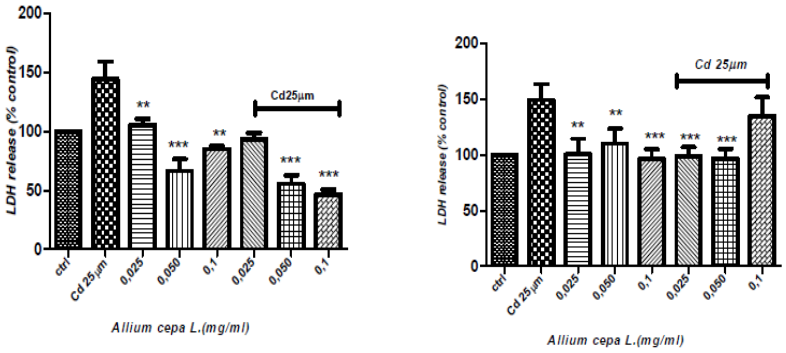


Fig 6. Lactate dehydrogenase enzyme activity of Caco-2 cell after 24 h(a) and 72 h (b) treatment with different concentrations of *Allium cepa L and* $CdCl_2$ (LDH assay). Data are expressed as mean ± SEM (n=3). Significant differences *versus* control ***$P < 0.05$). Come sopra per le figure ok

Potremmo uniformare, come di consueto, con $^{\#}p < 0.05$, $^{**}p < 0.01$, $^{\#\#\#}p < 0.001$?SI

4. Discussion

The present work investigated the capacity of the hydroalcoholic extract, rich in phenolics, from the outer scales of Tropea red onion (*A. cepa* L. var. Tropea) to protect Caco-2 cells against Cd induced toxicity. Due to its extremely long half-life, Cd represents a serious environmental health hazard causing a broad spectrum of toxicological effects especially to liver and kidney and there are some experimental evidences indicating that oxidative stress and reactive oxygen species generated in presence of Cd are involved in its toxic effects (Kim M.Y., Shon W-J, Park Mi-Na , Lee Y.S. and Shin D-Mi (2016) Protective effect of dietary chitosan on cadmium accumulation in rats. Nutrition Research and Practice;10(1):19-25). Plants and vegetables may absorb high levels of Cd from contaminated soil and thus the metal may also enter the food chain. Following oral exposure or ingestion, Cd first reaches and damages the intestinal epithelium.

Toxic effects associated with Cd have been widely studied in different biological systems (possiamo inserire qualche riferimento di quelli seguenti ??); in the present study, by investigating for the first

time the cytoprotective effects of the extract from the outer scales of Tropea red onion against Cd in Caco-2 cells taken as a model system for intestinal cells, we have revealed for the first time that this extract (0.025–0.1 mg/ml) was capable of blocking Cd-induced cell death.

Previous studies have shown that Cd exerts cytotoxic effects on the human liver cell line HL-7702 and the human intestinal cell line Caco-2 cells, inducing oxidative damage and apoptosis after 12h of treatment at Cd concentrations exceeding 0.5 mg L^{-1} (Rukhsanda Aziz M T Rafiq, Jie Yang, Di Liu, Lingli Lu, Zhenli He, M K Daud , Tingqiang Li, Xiaoe Yang 2014 Impact Assessment of Cadmium Toxicity and Its Bioavailability in Human Cell Lines (Caco-2 and HL-7702) *BioMed Research International Volume 2014, Article*). In another study, two hepatoma cell lines, HTC and HepG2 cells were treated with $CdCl_2$ and following 24h exposure to the heavy metal solution, the MTT assay indicated EC50 values of 100µM and 15µM for the two cell lines, respectively (George Fotakis, John A. Timbrell, 2006 In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride *Toxicology Letters 160 171–177)*. Furthermore, it has been observed that $CdCl_2$ at the concentration of 1–10 µM was able to induce apoptosis in H4IIE rat-derived hepatocyte cell lines after 12h to treatment (Kim SC, Cho MK, Kim SG. (2003) Cadmium-induced non-apoptotic cell death mediated by oxidative stress under the condition of sulfhydryl deficiency. *Toxicol Lett*; 144:325–36; Toxicity of Cd to Caco-2 cells was also confirmed by this study as shown by the MTT and LDH assay after 24 h exposure of the cells to the heavy metal.

A substantial number of studies have proven the efficacy of common onion, *A. cepa*, as a standard plant model to study cytotoxicity of natural products. *In vivo* experiments with an *A. cepa* aqueous extract (0.5- and 1-mL onion/100 g bw/day for 7 days) on Cd-intoxicated male Wistar rats suggested a chemoprotective capacity of this extract against Cd led to a significant and dose-dependent restoration of renal oxidants (lipid peroxidation and Glutathione-S transferase) and antioxidant (SOD, CAT and GSH) thus enhancing the antioxidant defense and reduction of the renal lipid peroxidation Suru S.M. (2008) Onion and garlic extracts lessen cadmium-induced nephrotoxicity in rats. *Biometals.* 2008 Dec;21(6):623-33.; Suru S.M.and Ugwu C.E., (2015) Comparative assessment of onion and garlic extracts on endogenous hepatic and renal antioxidant status in rat *J Basic Clin Physiol Pharmacol* Jul;26(4):347-54; Ola-Mudathir K.F., Suru S.M., Fafunso M.A., Obioha U.E., Faremi T.Y. (2008) Protective roles of onion and garlic extracts on cadmium-induced changes in sperm characteristics and testicular oxidative damage in rats. *Food and Chemical Toxicology* 46, 3604–3611) reported a protective effect of common onion on sperm and testicular oxidative damage induced by Cd in Wistar rats. Attenuation of the adverse effects were mediated by the reduction of testicular LPO, GST and MDA, and the increase of SOD, CAT and GSH. Another study also showed that treatment with Cd induced renal dysfunction in Wistar rats, while association of the heavy metal with an aqueous extract of *A. cepa* **(**1 mL/day), improved plasma and tissue levels of SOD, CAT and MDA Ige S.F., Salawu E.O., Olaleye S.B., Adeeyo O.A., Badmus J., Adeleke A.A. (2009) Onion *(Allium cepa)* extract prevents cadmium induced renal dysfunction. *Indian Journal of Nephrology* 19 (2009)140–144. In our study with Tropea red onion, we established that treatment of Caco-2 cells with the outer scales extract *plus* Cd for 24–72 h induced cell resistance to the heavy metal as assessed by the MTT (increase of cell viability) and LDH (reduction of enzyme release) assays. In particular, we observed that cell incubation also with the extract prevented the toxic effect induced by Cd alone with a remarkable effect already at the concentration of 50 µg/mL (Fig. 5). Qui

nella discussione non entrerei nel merito delle 24-72 ore (l'abbiamo già detto nei risultati). OK si può eliminare

The chemoprotective properties of plant extracts against Cd toxicity have been analyzed in a number of papers (Roopha DP and Padmalatha (2012) Effect of herbal preparation on heavy metal (cadmium) induced antioxidant system in female wistar rats, *J Med Toxicol* 8: 101-107;; Brzóska MM, Borowska S, Tomczyk M (2016) Antioxidants as a potential preventive and therapeutic startegy for cadmium. *Current drug Targets* 17:1350-1384; Mężyńska M and Brzóska MM (2018) Review of polyphenol-rich products as potential protective and therapeutic factors against cadmium hepatotoxicity.*J Applied Toxicol* 1-29) and mostly related to the high content of antioxidant compounds such as polyphenols. To the best of our knowledge, while the total content of polyphemols in the extract used in the bioassays is generally reported (Genchi G, Sinicropi MS, Lauria G, Carocci A, Catalano A (2020). The effects of cadmium toxicity. Int J Environmental Res Public Health, 17, 3782; doi:10.3390/ijerph17113782) the detailed chemical characterization of the extract is instead often lacking. In contrast, the activity of several pure phytochemicals as potential protective agents against Cd has been described (Renugadevi J and Prabu SM (2009). Naringenin protects against cadmium-induced oxidative renal dysfuntion in rats. Toxicology 256: 128-134; Mohajeri M, Rezaee M, Sahebkar A (2017). Cadmium-induced toxicity is rescued by curcumin:a review. BioFactors DOI 10.1002/biof.1376; Mężyńska M and Brzóska MM (2018) Review of polyphenol-rich products as potential protective and therapeutic factors against cadmium hepatotoxicity.J Applied Toxicol 1-29). As reported above (see Results), the polyphenolic composition of the extract from Tropea red onion used in our study was fully characterized and quercetin and quercetin glycosylated derivatives were identified as the main components. Quercetin with its water soluble glycosylated derivatives (Materska M. Quercetin and its derivatives: chemical structure and bioactivity- a review (2008) Pol J Food Nutr Sci 58:407–13; Adorisio S, Argentieri MP, Avato P, Caderni G, Chioccioli S, Cirmi S, Delfino DV, Greco G, Hrelia P, Iriti M, Lenzi M, Lombardo GE, Luceri C, Maugeri A, Montopoli M, Muscari I, Nanì MF, Navarra M, Gasperini S, Turrini E, Fimognari C (2021) The molecular basis

of the anticancer properties of quercetin. Pharmadvances 3: 496-520) plays a broad range of biological activities strictly due to its chemical structure. Generally, quercetin aglycone is a lipophilic molecule, whose hydrophilicity is increased by glycosylation. In addition, due to its catecholic nature can became oxidated and form a quite stable reactive ROS-scavenger or it can undergo auto-oxidation originating electrophilic bioactive quinones capable to bind to nucleophilic amino acid residues at active site of cell enzymes. Furthermore, quercetin polyhydroxylated structure enables the aglycone and its glycosylated derivatives to act as chelating agents to complex metals thus preventing the metal-mediated production of free oxidazing molecular species. Consistently, all these structural properties of quercetin and its coniugated derivatives well account for the high antioxidant activity displayed by our extract both in the DPPH and ß-carotene-linoleate assays as well as its capacity to inhibit NO release.

Quite interesting, incubation of Caco-2 cells with the Tropea red onion extract alone at the concentration of (0.025–0.1 mg/ml) for 24 h resulted in an increase of cell proliferation, suggesting that the cellular protein(s) responsible for cell survival are (is) activated by the components of the extract. In agreement with our finding, it has been reported in other studies that polyphenolic compounds might produce beneficial and/or cytotoxic action *in vitro* through their capacity to stimulate or modulate the antioxidant defense system in different cell lines. The different response, ranging from no effect/proapoptotic/survival/proliferative effect, has been correlated with their distinct chemical features and the type of cell line employed (Ramos S, Rodríguez-Ramiro I, Martín MA, Goya L, Bravo L (2011) Dietary flavanols exert different effects on antioxidant defences and apoptosis/proliferation in Caco-2 and SW480 colon cancer cells. *Toxicology in vitro*, 25: 1771-1781). The proliferative effect of the Tropea red onion extract on Caco-2 cells may also be correlated with the cytoprotective effect against Cd induced cell injury as assessed by the treatment of the adenocarcinoma cells with both the extract and the heavy metal solution. Studies on heavy-metal toxicology showed that Cd-induced toxicity results from protein binding of metals and metal–protein interactions. (Fatma M El-Demerdash, Mokhtar I Yousef, Fatma S Kedwany, Hoda H Baghdadi.,

2004 Cadmium-induced changes in lipid peroxidation, blood hematology, biochemical parameters and semen quality of male rats: Protective role of vitamin E and β-carotene *Food and Chemical Toxicology 42 (2004) 1563–1571).* Nevertheless, it has been demonstrated that metal-flavonoid complexes with a lower redox potential have a higher antioxidant potency than the free aglycones and in addition the metal-ligand interaction may improve their bioavailability (Materska (2008); Treml J, Šmejkal K. Flavonoids as potent scavengers of hydroxyl radicals (2016). Compr Rev Food Sci Food Saf 15:720–38; Rodrigues-Arce E and Saldias m (2021) Antioxidant properties of flavonoid metal complexes and their potential inclusion in the development of novel strategies for the teratment against neurodegenerative diseses.Biomedicine & Pharmacotherapy 143, 112236). On this basis and from the present results, we would suggest that the protective activity of Tropea red onion extract against Cd, observed in our study, derives from an enhanced antioxidant activity due to a metal complexation of the active components of the extract.

Overall, results obtained with Caco-2 cells, representing a model of the intestinal barrier, can be combined with those from our previous study aimed to investigate the mechanism of action of Tropea red onion extract by using planar lipid membranes surrogate of intestinal membranes (Marrelli et al, 2021). The electrophysiological study proved that the extract was able to induce ionic flux across the planar membranes and form channel-like events affecting their biophysical properties. In light of the results obtained, it is possible to hypothesize that the extract, interacting with the lipid bilayer, protects the lipids from the oxidative deterioration induced by the Cd.

XXXX Vorrei fare un aggancio al lavoro precedente: dalla letteratura sembra che Cd possa interferire con la fosfolipasi: chiedo a Daniela e Rosanna se hanno una spiegazione per legare questi risultati si Caco-2 e quelli precedent sulle PLM. GRAZIE

Formattato: Tipo di carattere: Non Grassetto, Italiano (Italia), Evidenziato

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Formattato: Tipo di carattere: Non Grassetto, Inglese (Regno Unito), Evidenziato

5. Conclusions

In summary, our preliminary study suggests that the outer scales extract from Tropea red onion, rich in phenolics, exerts a cytoprotective action against Cd induced damage. Consistently, our work shows that Tropea red onion extract has the ability to minimize the toxic effects of Cd in a dose- and time-dependent manner. Further studies are however very important to understand if the representative major phenolics are responsible for the cytoprotective effects of this extract. We aim to accomplish future studies to deeper investigate the cytoprotective effects of Tropea red onion against apoptotic and non-apoptotic cell death induced by Cd and gather some information on the mechanistic basis of its cytoprotective effects.

Disclosure statement

No potential conflict of interest was reported by the authors.

Acknowledgments

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