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A simple liposome assay for the screening of zinc ionophore activity of polyphenols

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ABSTRACT

An efficient liposomal system for screening the zinc ionophore activity of a selected library consisting of the most relevant dietary polyphenols is presented. The zinc ionophore activity was demonstrated by exploring the use of zinc-specific fluorophore FluoZin-3 loaded liposomes as simple membrane tools that mimic the cell membrane. The zinc ionophore activity was demonstrated as the capacity of polyphenols to transport zinc cations across the liposome membrane and increase the zinc-specific fluorescence of the encapsulated fluorophore FluoZin-3. In addition, the zinc chelation strength of the polyphenols was also tested in a competition assay based on the fluorescence quenching of zinc-dependent fluorescence emitted by zinc–FluoZin-3 complex. Finally, the correlation between the chelation capacity and ionophore activity is demonstrated, thus underlining the sequestering or ionophoric activity that the phenolic compounds can display, thus, providing better knowledge of the importance of the structural conformation versus their biological activity. Furthermore, the assays developed can be used as tools for rapid, high-throughput screening of families of polyphenols towards different biometals.

1. Introduction

Zinc ions have long been known to mimic the actions of hormones, growth factors, neurotransmitters and cytokines, and it is believed that zinc may act on intracellular signaling molecules (Beyersmann & Haase, 2001; Colvin, Fontaine, Laskowski, & Thomas, 2003; Frederickson, 2003). In fact, zinc is a known inhibitor of protein tyrosine phosphatases (Brautigan, Bornstein, & Gallis, 1981) with a constant of inhibition in the nanomolar range (Maret, Jacob, Vallee, & Fischer, 1999). In addition, zinc affects the regulation of transcription factors, and can induce the expression of some genes, including those coding for molecules involved in zinc homeostasis, such as zinc transporters and metallothioneins (Palmiter & Huang, 2004). The gene expression of metallothioneins by zinc is regulated by metal response element-binding transcription factor-1 (Lichtlen & Schaffner, 2001). The chemical properties of zinc that differentiate it from other transition metals, such as copper and iron, which display several different oxidation states in biological systems, is that zinc exists as a redox inert Zn^{2+} cation, which does not undergo redox reactions at physiological redox potentials (Eide, 2011; Laitaoja, Valjakka, & Jänis, 2013). Additionally, zinc can induce the expression and maintain the levels of potential radical scavenging proteins such as metallothionein (MT), the major zinc binding protein associated with zinc homeostasis (Quesada et al., 2011), DNA protection, oxidative stress, and apoptosis (Higashimoto et al., 2009; Tapiero & Tew, 2003). Furthermore, it can act through stabilization of cell membranes (Powell, 2000) or as a structural component of anti-oxidant enzymes (Klotz, Kröncke, Buchczyk, & Sies, 2003).

On the other hand, recent studies have focused on dietary phenolic compounds as natural improvers of health and more than 8000 dietary polyphenols have been identified (Araújo, Gonçalves, & Martel, 2011). The growing interest in these compounds resides in the accumulating evidence regarding their ability to trigger several cellular pathways leading to the prevention and/or amelioration of pathological conditions, acting as antioxidants (Leopoldini, Russo, & Toscano, 2011), anti-carcinogenics (Ramos, 2008; Čipák, Rauko, Miadoková, Čipáková, & Novotný, 2003), anti-inflammatories (Bravo, 1998), neuroprotectors (Russo,

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Spagnuolo, Tedesco, Bilotto, & Russo, 2012), anti-lipidemic and vaso-relaxing agents (Araújo et al., 2011).

In recent decades, it has been demonstrated and understood that phenolic compounds interact with different metals, including zinc, and because of their distinctive chemical structure, they can easily form complexes through metal ion chelation (Hider, Liu, & Khodr, 2001) in a manner similar to that of other well-known metal chelators, such as the drug clioquinol (CQ), and also exert ionophore activity comparable to pyrithione (Pyr) (Cao et al., 2013, 2014). The first evidence of polyphenol–metal complexes was reported in 1970 between aluminum ions and flavonoids (Porter & Markham, 1970). Since then, more than 40 metal–flavonoid complexes have been investigated (Grazul & Budzisz, 2009).

One of the mechanisms by which flavonoids exert their antioxidant activity is via the chelation of redox-active transition metals (Thompson, Williams, & Elliot, 1976), which are known to catalyze many biological processes leading to the production of free radicals (Mladenka, Zatloukalová, Filipský, & Hrdina, 2010). The essential sites for metal chelation are hydroxyl groups, and the most suitable cations for chelation are Fe(II), Fe(III), Cu(II) and Zn (II) as they have high charge density, stimulating the interaction with the phenoxide groups that have a high negative charge density (Hider et al., 2001). The structure of the complexes formed depends on the type of flavonoid and metal ion involved, which in turn can influence its biological interactions that may be different from the native flavonoid (Afanas'eva, Ostrakhovitch, Mikhal'chik, Ibragimova, & Korkina, 2001; Aherne & O'Brien, 2000; Fernandez, Mira, Florêncio, & Jennings, 2002; Mira et al., 2002). Depending on the polyphenol and its potential binding sites, different structures could be formed with different stoichiometries, thus affecting the biological function of the complex (Wei & Guo, 2014). Experimental data has indicated that the chelated compounds are more effective free radical scavengers than flavonoids alone, suggesting that the Zn–polyphenol complexes not only exert singular biological properties, but can also enhance the effects of both compounds individually (Selvaraj, Krishnaswamy, Devashya, Sethuraman, & Krishnan, 2014).

Further studies have revealed that polyphenols not only interact with metal ions, but also deeply modulate expression of MTs, cellular zinc transporters, extracellular zinc carriers, and intracellular zinc accumulation which are key factors in zinc homeostasis (Quesada et al., 2011). Zinquin is a fluorescent zinc-specific indicator and an increase in zinquin-detectable cytoplasmic levels of zinc in a HepG2 cell line has been monitored when treated with phenolic compounds (Quesada et al., 2011). This increment in intracellular zinc levels has been reported to induce apoptosis of tumor cells (Ding, Liu, Vaught, Yamauchi, & Lind, 2005; Feng, Li, Guan, Franklin, & Costello, 2008), suggesting that zinc ionophores may serve as anticancer agents (Liang et al., 1999).

Although the ionophore activity of naturally occurring compounds has not been well established, there is strong evidence of their interaction and complex formation with zinc ions (Selvaraj et al., 2014), suggesting that they could be potential candidates as zinc ionophore molecules. The interaction of quercetin (QCT) and epigallocatechin-3-gallate (EGCG) with zinc, as well as their ionophore activity has been confirmed in a liposome model using the specific zinc indicator FluoZin-3 (Dabbagh-Bazarbachi et al., 2014). Luteolin (LUT) and naringenin (NAR) interact with zinc ions, forming complexes and exerting a biological function acting as strong radical scavengers (Chen, Wu, & Li, 2009; Wang, Yang, & Wang, 2006). The ability of genistein (GEN) to bind zinc ions has not been well elucidated, although its ability to bind iron is well known and these complexes exert a strong anti-oxidant role, and this suggests that it could have a similar action with other metals such as zinc (Harper, Kerr, Gescher, & Chipman, 1999). There is also evidence regarding the ability of catechin hydrate (CAT HYD), which is the one of the main bioactive components in green tea, to interact and form complexes with zinc ions (Bodini, del Valle, Tapia, Leighton, & Berrios, 2001; Le Nest et al., 2004), exerting an anti-oxidant activity, but also having an essential role in the treatment of different cancers, such as prostate cancer (Yu, Shen, & Yin, 2007). Several reports have confirmed that rutin (RUT) forms complexes with zinc (Bai et al., 2004), also by acting as a free radical scavenger, more effectively than the free flavonoid (De Souza & De Giovani, 2004). The anti-inflammatory activity of this bioflavonoid is also enhanced when complexed with zinc (Afanas'eva et al., 2001). Taxifolin (TAX) is also able to interact and form complexes with zinc ions, also acting as an effective radical scavenger (Donracheva et al., 2009). Most phenolic acids are good metal chelators, due to their structure with several catechol and/or galloyl moieties (Andjelkovic et al., 2006). To our knowledge, there are no reports to date on interactions and complex formation with zinc ions with phloretin (PHLO) or the stilbene resveratrol (RSV), although for RSV there is evidence regarding complex formation with copper, suggesting that potentially similar structures can be formed with other metal ions (Chiavarino et al., 2012). Catechol (CAT) is one of the simplest naturally occurring polyphenols, and also one of the most important moieties in a high variety of polyphenols, responsible for the interaction with metal ions. CAT forms complexes with Ruthenium, a rare transition metal, suggesting that it could have the same behavior with other transition metals like zinc (Almeida et al., 2007). Thus, a high proportion of polyphenols present some kind of interaction with zinc or other metal ions, although for the majority of polyphenols the ionophore activity is still undescribed.

The aim of this work was to evaluate the capacity of fourteen different phenolic compounds to bind and chelate zinc ions in solution and to demonstrate their ability to act as zinc ionophores. We focused on fourteen phenolic compounds grouped according to their chemical structure, including the flavonoids quercetin (QCT), epigallocatechin-3-gallate (EGCG), luteolin (LUT), naringenin (NAR), phloretin (PHLO), genistein (GEN), catechin hydrate (CAT HYD), rutin (RUT) and dihydroquercetin or taxifolin (TAX); the phenolic acids gallic acid (GAL), tannic acid (TAN) and caffeic acid (CAF); the stilbene resveratrol (RSV); and other polyphenols such as catechol (CAT). Two different zinc ionophore agents, clioquinol and pyrithione, were used to compare the ionophore activity of the selected polyphenols, as well as the zinc sequestrant molecule, TPEN (N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine). The binding/chelation of the zinc ions by the polyphenols was evaluated using a competition assay based on the fluorescence quenching of zinc-dependent fluorescence emitted by FluoZin-3. In this competition assay the zinc chelation strength of each phenolic compound was correlated with the decrease in the fluorescence signal due to the dissociation of the zinc–FluoZin-3 complex as zinc cations are sequestered from the fluorophore complex by the polyphenol. In addition, we present a simple and rapid liposome assay for demonstrating the zinc ionophore activity of common dietary polyphenols. The method exploits the use of unilamellar liposomes loaded with the zinc-sensitive fluorophore FluoZin-3 as simple membrane models that mimic biological cell membranes to monitor the capacity of the phenolic compounds to transport zinc cations across the lipid bilayer. The zinc ionophore activity presented by the bioactive nutrients was compared with the strong, well-established synthetic pharmacological ionophores, such as clioquinol and pyrithione. The correlation between the chelation capacity and ionophore activity underlines the different behaviors that the phenolic compounds can display and the liposomal assays developed can be used as tools for the rapid, high-throughput screening of ionophore activity of families of polyphenols.

2. Experimental section

2.1. Materials

All the phenolic compounds, pyrithione (PYR), quercetin (QCT), epigallocatechin-3-gallate (EGCG), genistein (GEN), taxifolin (TAX), luteolin (LUT), phloretin (PHLO), catechol (CAT), naringenin (NAR), rutin (RUT), catechin (CAT HYD), caffeic acid (CAF), tannic acid (TAN), gallic acid (GAL), resveratrol (RSV) and clioquinol (CQ) were purchased from Sigma–Aldrich, as well as the lipid 1,2-dipalmi toyl-sn-glycero-3-phosphocholine (DPPC), cholesterol, dimethyl sulfoxide (DMSO), ethanol, zinc chloride ($ZnCl_2$), N,N,N′,N′-tetra kis(2-pyridylmethyl)ethylenediamine (TPEN) and the phosphate buffered saline (0.01 M PBS, pH 7.4). Cell impermeant FluoZin-3 tetrapotassium salt was obtained from Molecular Probes. A Simplicity 185 Millipore-Water System was used to obtain Milli-Q water (18.2 $m\Omega\,cm^{-1}$) for the preparation of buffers and liposomes. The compounds PYR, QCT, EGCG, GEN, TAX, LUT, PHLO, CAT, NAR, RUT, CAT HYD, CAF, TAN, GAL, RSV and CQ were dissolved as 100 μM solutions in 100% DMSO and stored at −20 °C. $ZnCl_2$ was stored as 1 M solution in ethanol/PBS (50/50% v/v). FluoZin-3 zinc indicator was used at 10 μM in 100% DMSO.

2.2. Measurement of the interaction of the polyphenols with zinc cations in solution

A competition assay was carried out to test the ability of the polyphenols PYR, QCT, EGCG, GEN, TAX, LUT, PHLO, CAT, NAR, RUT, CAT HYD, CAF, TAN, GAL, RSV and CQ to chelate zinc cations in solution, thus reverting their binding with the zinc specific fluorophore FluoZin-3. TPEN was also included as a positive control, to compare with a well-recognized zinc chelator (Blindauer, Razi, Parsons, & Sadler, 2006). FluoZin-3 tetrapotassium salt was used to demonstrate the capacity of the flavonoids and of clioquinol to form complexes with zinc. First, a fluorescence titration curve was constructed using a range of zinc concentrations (from 0 to 20 μM) to allow us to select a concentration of zinc cations at which 3 μM FluoZin-3 fluorescence is not saturated. The relative capacity of the polyphenols, clioquinol and TPEN to quench the zinc-dependent fluorescence of 3 μM FluoZin-3 at 1 μM zinc concentration was then monitored. The final concentration of test substances in the competition reaction with FluoZin-3 was 10 μM.

Briefly, 3 μM FluoZin-3 was mixed with 1 μM $ZnCl_2$ (final concentrations) in PBS (0.01 M, pH 7.4) and incubated for 15 min at room temperature to facilitate formation of the zinc complex prior to measuring the fluorescence intensity. Subsequently, 10 μM (final concentration) of the test substances (PYR, QCT, EGCG, GEN, TAX, LUT, PHLO, CAT, NAR, RUT, CAT HYD, CAF, TAN, GAL, RSV, CQ or TPEN), were added to each cuvette respectively, mixed vigorously and incubated at 37 °C for 30 min under shaking conditions and protected from light, before monitoring the fluorescence. All fluorescence measurements were performed in an Eclipse fluorescence spectrophotometer from Varian coupled with a Cary temperature controller at 25 °C using quartz cuvettes with 1 cm path length and with a maximum volume of 150 μL. The excitation and emission wavelengths used were 494 nm and 516 nm with slits of 5 nm.

2.3. Preparation of FluoZin-3 loaded liposomes

Liposomes were prepared using the curvature-tuned method previously reported (Genç, Ortiz, & O'Sullivan, 2009). Briefly, FluoZin-3 (final concentration 3 μM) was mixed with 2 mL of PBS (0.01 M, pH 7.4) in a glass reactor protected from light-induced degradation, under stirring conditions and bubbling argon gas. After 15 min, a previously homogenized mixture of DPPC and cholesterol (9:1 molar ratio) in 3 mL PBS was added and maintained under stirring conditions and argon at 25 °C for another 15 min. The homogeneous mixture was then subjected to a rapid pH jump from pH 7.4 to pH 11 by pipetting a previously calculated amount of NaOH, and then readjusted to pH 7.4 using HCl within a 3 s frame, followed by an equilibration step of 25 min where lipid clusters curl into liposomes entrapping the buffer containing the FluoZin-3 molecules. The resulting FluoZin-3-loaded liposomes were purified to remove any unencapsulated material by size exclusion chromatography (SEC) using a Sephadex G-100 column and the size and charge of the formed liposomes was determined using dynamic light scattering (DLS) and Zeta-potential. Prepared liposomes were used immediately.

2.4. Liposome assay to assess zinc ionophore activity

The zinc ionophore activity of the different polyphenols, clioquinol and pyrithione was demonstrated by the increase in zinc-dependent fluorescence of FluoZin-3 loaded liposomes. Freshly prepared liposomes loaded with FluoZin-3 were placed in separate vials and their fluorescence measured. Subsequently, $ZnCl_2$ was added to each solution to a final concentration of 10 μM, the solution was softly vortexed, incubated at 25 °C for 30 min and the fluorescence was measured again. Finally, 50 μM (final concentration) of PYR, QCT, EGCG, GEN, TAX, LUT, PHLO, CAT, NAR, RUT, CAT HYD, CAF, TAN, GAL, RSV or CQ was added to each vial respectively, softly vortexed and allowed to incubate at 25 °C for 30 min under shaking conditions before measuring their fluorescence. The evaluation of the ionophore behavior of each polyphenol was tested in a time-dependent assay to further understand the velocity of the zinc transport. The kinetic experiment was carried out by continuously measuring the fluorescence of the FluoZin-3 loaded liposomes over a period of time of 70 min, with the addition of $ZnCl_2$ (final 10 μM) after 5 min and the addition of the test substances (final 50 μM) to each cuvette respectively after 10 min. All fluorescence measurements were performed in an Eclipse fluorescence spectrophotometer from Varian coupled with Cary temperature controller at 25 °C using quartz cuvettes with a 1 cm path length and with a maximum volume of 150 μL. The excitation and emission wavelengths used were 494 nm and 516 nm with slits of 5 nm. Control experiments were performed by adding to the cuvette FluoZin-3 loaded liposomes with 10 μM $ZnCl_2$ and the solvent used to dissolve the ionophores (final DMSO concentration 0.1% v/v) after 10 min.

3. Results and discussion

3.1. Zinc chelation strength of polyphenols in solution

Several polyphenols have been widely reported to chelate metals through their deprotonated hydroxyl groups, in which the oxygen possesses a high charge density offering a strong ligand for metal-binding. As expected, the chelation strength depends on the number of hydroxyl ligands, but also on their proximity, thus bi- or poly-dentate ligands are stronger scavengers than monodentate ligands. A detailed structure of the identified chelating groups of each of the polyphenols tested in this work, as well as their classification and food source, is presented in Supporting information S1.

In order to quantify the relative capacity of the phenolic compounds tested in the work reported here (QCT, EGCG, GEN, TAX, LUT, PHLO, CAT, NAR, RUT, CAT HYD, CAF, TAN, GAL and RSV), in addition to the established ionophore (CQ and PYR) and sequestrant (TPEN) agents, to bind zinc cations in aqueous solutions at

physiological pH, their capacity to retrieve zinc cations from FluoZin-3–zinc complexes was measured, by monitoring the decrease in zinc-dependent fluorescence emitted by the FluoZin-3–zinc complexes upon addition of the polyphenols to the solution.

Firstly, a fluorescence titration assay was carried out showing that the fluorescence of FluoZin-3 (final concentration of 3 μM) increases with the addition of zinc cations up to ~3 μM Zn^{2+}, as expected from its 1:1 complex ratio, where a plateau is reached and FluoZin-3 fluorescence saturates (data not shown). Therefore, a zinc concentration of 1 μM was selected as optimal, at which all the zinc is bound to FluoZin-3 and not in a free form due to fluorophore saturation, to demonstrate the decrease in fluorescence of 3 μM FluoZin-3–Zn complexes upon addition of the phenolic compounds. As can be seen in Fig. 1, all 14 polyphenols, together with clioquinol, pyrithione and TPEN, at 10 μM concentrations, resulted in a decrease in the zinc-dependent FluoZin-3 fluorescence to some extent, due to sequestering of the zinc ions. The highest zinc-chelating strength was observed to be produced by TPEN, followed by CQ, with a complete decrease of the FluoZin-3 fluorescent signal. Similarly, PYR caused an almost 80% reduction of the fluorescence signal. These observations are anticipated, as they are well-known powerful zinc chelators. Regarding the phenolic compounds, TAN surprisingly also quenched 100% of the fluorescence signal, which can be explained by its complex molecular structure having 25 hydroxyl groups, most of them positioned in a powerful bi-dentate configuration (Supporting information S1), ready to bind and sequester zinc cations from the fluorescent zinc–FluoZin-3 complex. Regarding the rest of the polyphenols, the fluorescence quenching capacity was smaller and similar, within a range from 30% to 15%, and in agreement with the number and position metal-binding sites present on each compound. For example, the flavonoids having –OH groups positioned together offering a bi-dentate ligand (QCT, EGCG, LUT, GAL, TAX) instead of having the –OH groups positioned on different sides of the molecule (NAR, PHLO, GEN), were observed to have a higher chelating strength. However, containing more hydroxyl groups alone does not result in improved chelation. As it was observed there are other important factors affecting the chelating strength, including the three-dimensional conformation of the potential binding groups, as well as the stoichiometry formed between the polyphenols and the metal. Therefore, the structure of the polyphenols studied does not always correlate with their chelating efficiency. The very low capacity of some of the polyphenols tested to quench the FluoZin-3 fluorescence despite containing several –OH groups, as well as some compounds observed to have a high ability to chelate zinc ions, whilst only possessing few hydroxyl groups can thus be attributed to their 3-D conformation and stoichiometry.

3.2. Zinc ionophore activity of polyphenols

Polyphenols are known to interact with lipid bilayers and actively modify their membrane fluidity (Arora, Byrem, Nair, & Strasburg, 2000; Saija et al., 1995; Yu, Chu, Hagerman, & Lorigan, 2011). It is believed that the fluidization of the bilayer is due to the intercalation of the lipophilic domains of the molecules within the ordered structure of the lipid membranes. This interaction strongly depends on several characteristics of the polyphenolic molecule, such as its degree of hydroxylation and their stereochemistry, the polarity and the 3-D structural features. However, the transport phenomena of molecules across lipid membranes is still not fully understood. The permeabilization of the membrane to low-molecular-weight molecules by ionophore molecules has been suggested to also be dependent on the concentration of monovalent ions thus creating a gradient of ions and modifying the membrane potential, thus inducing its depolarization (Alonso & Carrasco, 1980, 1982). In addition, several other factors may play an important role and modulate the ionophore strength, such as the type of ion-ionophore complex formed, the different ratios, the kinetic reaction of complexation/decomplexation, the ion-ionophore membrane interaction as well as its transmembrane diffusion constant (Erdahl, Chapman, Wang, Taylor, & Pfeiffer, 1996). For example, Yang et al. (2009) reported that the behavior of EGCG was modified due to the formation of zinc–EGCG complexes, resulting in an enhancement of the incorporation of EGCG into the liposome membrane, which could cause the formation of ion passages.

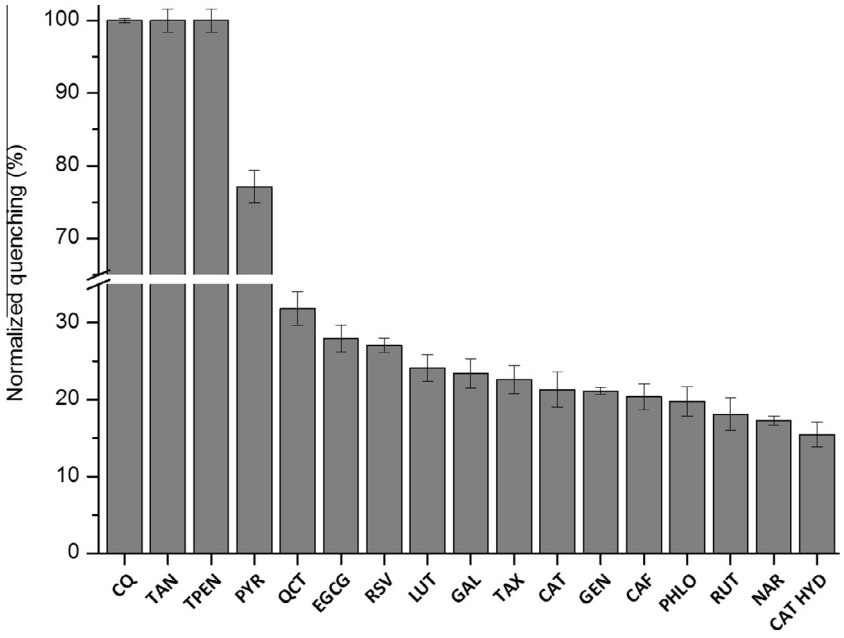

Fig. 1. Chelation strength of zinc cations by the polyphenols, clioquinol, pyrithione and TPEN in solution. The quenching of the zinc-dependent fluorescence of FluoZin-3 indicates the capacity of the compounds at 10 μM to retrieve zinc cations from the zinc–FluoZin-3 complex formed between 3 μM FluoZin-3 and 1 μM $ZnCl_2$. All values are means ± SD of three independent experiments.

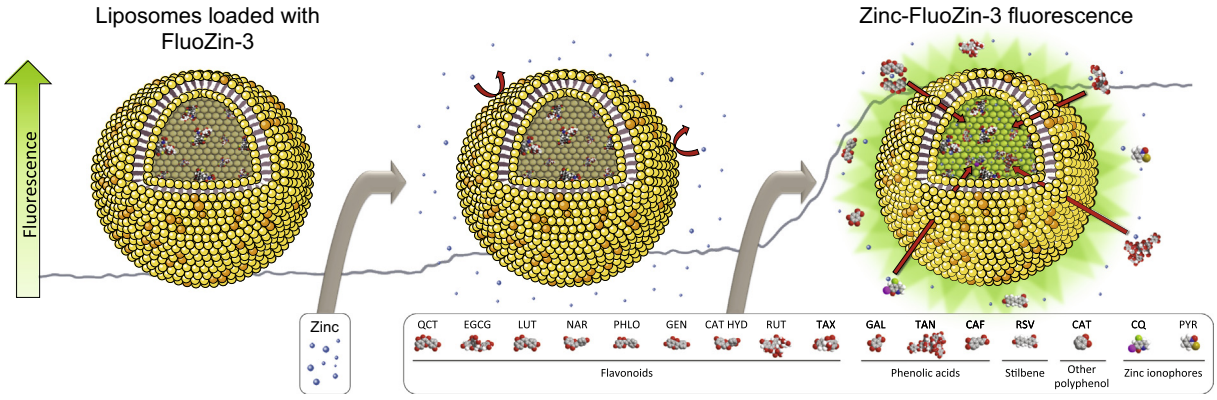

Fig. 2. Concept design of the liposome system for the zinc ionophore activity determination.

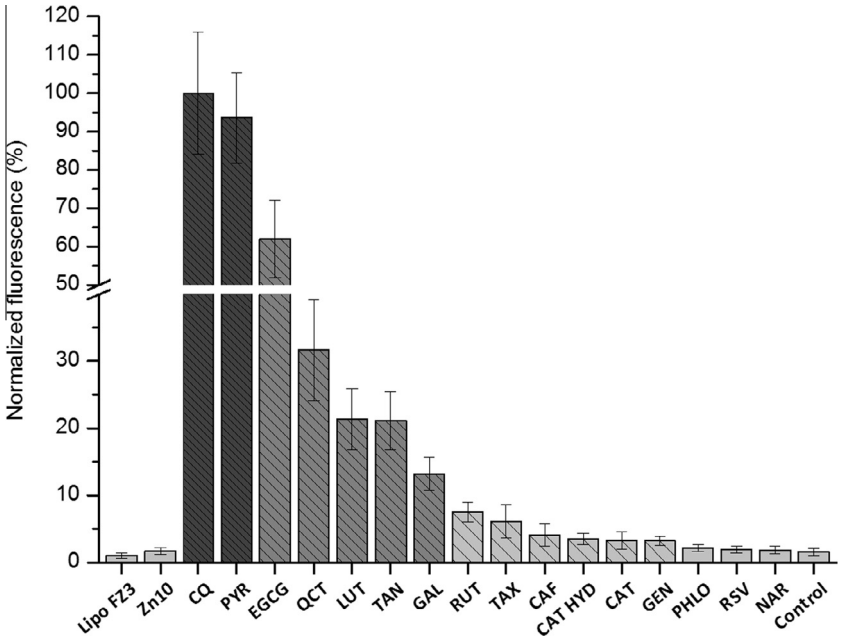

Fig. 3. Liposome assay for the determination of zinc ionophore activity of polyphenols, clioquinol and pyrithione. The increase in the zinc-dependent fluorescence of FluoZin-3 indicates the capacity of the compounds at 50 μM to interact with 10 μM zinc cations, transport them across the liposome membrane and present them to 3 μM FluoZin-3 in the liposome inner cavity. Control are FluoZin-3 loaded liposomes in the presence of 10 μM zinc cations with 0.1% DMSO. FluoZin-3 loaded liposomes in the absence of zinc (Lipo-FZ3) and in the presence of 10 μM $ZnCl_2$ (Zn10). λ_{ex} 494 nm/λ_{em} 516 nm. All values are means ± SD of three independent experiments.

A wide number of publications have reported the use of liposomes as simple membrane systems for the demonstration of ion transport across lipid bilayers (Kolber & Haynes, 1981; Mathew, Nagaraj, & Balaram, 1982; Weissmann, Anderson, Serhan, Samuelsson, & Goodman, 1980). We have previously reported the use of a liposomal system to study the zinc ionophore properties of QCT and EGCG (Dabbagh-Bazarbachi et al., 2014), and we also investigated that the flavonoid compounds did not induce the release of the liposome load, as was also reported by Ollila, Halling, Vuorela, Vuorela, and Slotte (2002).

A simple liposome system with zinc-dependent fluorophore FluoZin-3 encapsulated in the inner cavity was used to determine if the most commonly abundant polyphenols present in our diet can transport zinc across the cell membrane (Fig. 2). Using the liposome also limited the transport pathway to be solely due to transmembrane transport as no other transduction mechanisms normally present in cells would contribute to the transport of the zinc cations.

The zinc ionophore effect was extrapolated as a function of the increase in fluorescence due to the capacity of the polyphenols to carry zinc cations across the liposome membrane to interact with the encapsulated FluoZin-3. Liposomes with a mean size of 1.3 ± 0.2 μm and a net charge of 0.8 ± 0.2 mV loaded with FluoZin-3 before and after the addition of 10 μM $ZnCl_2$ showed a negligible fluorescence signal due to the impermeability of the DPPC:cholesterol liposome membrane at 25 °C to zinc cations. The subsequent addition of the phenolic compounds, caused an increase in the fluorescence signal due to the zinc complexation, transport and consequent interaction with FluoZin-3. The well-reported CQ and PYR zinc ionophores, resulted in a marked increase in the fluorescence signal, as expected, whilst each of the other phenolic compounds studied presented very different ionophore properties (Fig. 3). In the case of EGCG and QCT, both presented a notable zinc ionophore activity, EGCG > QCT, as previously reported by Dabbagh-Bazarbachi et al. (2014). In agreement with the results obtained in the chelation assay, the fluorescence increment (Δ) observed, can be clearly correlated with their chelation strength towards zinc. The polyphenols analyzed can be classified into three groups: strong, soft and no zinc ionophore activity. The polyphenols exhibiting a strong ionophore activity include EGCG (36-fold Δ), QCT (18-fold Δ), LUT (12-fold Δ), TAN (12-fold Δ) and GAL (8-fold Δ); those displaying a soft zinc ionophore

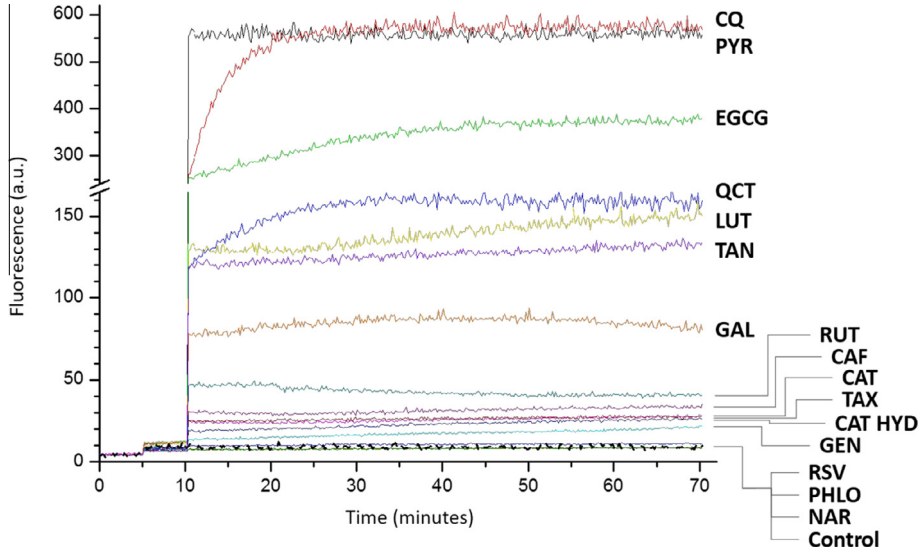

Fig. 4. Time-dependent fluorescence emission of FluoZin-3 loaded liposomes before (minute 0) and after (minute 5) addition of 10 μM $ZnCl_2$. The fluorescence increased upon the addition of the compounds (50 μM) at minute 10, and the fluorescence was monitored over the period of an hour. λ_{ex} 494 nm/λ_{em} 516 nm. Results are representative of at least three experiments. In the control sample, only the solvent used to dissolve the ionophores (0.1% DMSO) was added at minute 10 without showing any fluorescent increase. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

activity were RUT (4-fold Δ), TAX (4-fold Δ), CAF (3-fold Δ), CAT HYD (2-fold Δ), CAT (2-fold Δ) and GEN (2-fold Δ), whilst those resulting in no fluorescence were RSV, PHLO and NAR, indicative of a complete lack of zinc ionophore activity. In addition, control experiments demonstrated that the addition of a final concentration of 0.1% DMSO, as present in the tested samples, did not affect or destabilize the liposomes, thus keeping the FluoZin-3 within the liposomes and not resulting in an increase in fluorescence.

An evaluation of the kinetics of the ionophore behavior of each polyphenol was tested in a time-dependent assay to further understand the zinc transport strength (Fig. 4). The results showed that the increase of the zinc-FluoZin-3 fluorescence produced by PYR (Fig. 4, black line), until it reached maximum fluorescence, was instantaneous. CQ (Fig. 4, red line) also produced a very high increase in the fluorescence and rapidly achieved the maximum fluorescence reaching equilibrium in less than 15 min. Regarding the kinetics of the polyphenols studied, almost none of the ionophore active compounds showed a markedly time-dependent increase of the fluorescence. Only in the case of EGCG and QCT a plateau was reached after ca. 40 and 20 min respectively, exhibiting a slower, but efficient, chelation and transport kinetics. In addition, control experiments carried out by adding the different compounds to the FluoZin-3 loaded liposomes in the absence of $ZnCl_2$ did not show any increase in the fluorescence signal (data not shown).

In order to confirm the stability of all the liposomes immediately after the fluorescent experiments, dynamic light scattering (DLS) and Zeta potential analysis of the vesicles were carried out and the results are presented in Table 1. The DLS results confirmed the presence of stable liposomes that had not leaked the fluorophore following exposure to the polyphenols, maintaining roughly the same size as compared with the starting FluoZin-3 loaded liposomes (1.3 ± 0.2 μm) or the control samples (FluoZin-3 loaded liposomes with 10 μM $ZnCl_2$ at final 0.1% v/v DMSO) (1.1 ± 0.2 μm) in the absence of the tested compounds. The surface charge of the liposomes following exposure to the polyphenols also demonstrated that the main net charge of the FluoZin-3 loaded liposomes (0.8 ± 2.2 mV) was not significantly affected by the zinc-complexes as all measurements indicated approximately a zero charge. Both size and charge results confirmed that the liposome vesicles were maintained intact and the fluorescence signal was solely due to the transport of the zinc-ionophore complex through the lipid bilayer.

Table 1
Dynamic light scattering and Zeta-potential measurements of the liposomes loaded with FluoZin-3, as well as the fluorescence increment (Δ) caused by each compound, after the treatment with 10 μM $ZnCl_2$ and polyphenols, clioquinol, pyrithione and TPEN at 50 μM (0.1% DMSO final concentration). Standard deviations were calculated from the mean data of a series of experiments ($n \geqslant 3$).

Compound	Δ Fluorescence[a]	Liposome parameters	
		Size (μm)	Charge (mV)
Clioquinol (CQ)	57.2	1.3 ± 0.2	2.4 ± 2.9
Pyrithione (PYR)	53.6	1.4 ± 0.1	0.8 ± 1.1
Epigallocatechin-gallate (EGCG)	35.5	1.3 ± 0.1	1.6 ± 2.5
Quercetin (QCT)	18.1	1.3 ± 0.1	2.1 ± 1.8
Luteolin (LUT)	12.2	1.4 ± 0.3	1.1 ± 0.8
Tannic Ac. (TAN)	12.2	1.2 ± 0.1	−2.1 ± 3.6
TPEN	10.4	1.1 ± 0.2	0.0 ± 2.6
Gallic Ac. (GAL)	7.5	1.3 ± 0.3	−3.5 ± 2.8
Rutin (RUT)	4.3	1.6 ± 0.3	−2.0 ± 3.3
Taxifolin (TAX)	4.3	1.0 ± 0.3	3.6 ± 3.0
Caffeic Ac. (CAF)	2.5	1.4 ± 0.4	3.6 ± 1.6
Catechin (CAT Hyd)	2.4	1.2 ± 0.2	3.3 ± 0.6
Catechol (CAT)	1.9	1.4 ± 0.3	2.1 ± 1.3
Genistein (GEN)	1.8	1.4 ± 0.4	−2.1 ± 2.2
Phloretin (PHLO)	1.3	1.4 ± 0.2	2.6 ± 1.1
Resveratrol (RSV)	1.1	1.3 ± 0.2	−1.5 ± 4.1
Naringenin (NAR)	1.1	1.3 ± 0.2	4.6 ± 1.2
Control[b]	0.9	1.1 ± 0.2	−2.1 ± 3.1

[a] Increment of fluorescence is calculated by the signal obtained from the FluoZin-3 loaded liposomes in the presence of 10 μM $ZnCl_2$ and the respective compound at 50 μM (or solvent vehicle in the control) divided by the signal obtained from the FluoZin-3 loaded liposomes with 10 μM $ZnCl_2$.

[b] Control contains the FluoZin-3 loaded liposomes with $ZnCl_2$ (10 μM) in the solvent vehicle (0.1% DMSO).

The compounds analyzed have been demonstrated to interact to different extents with zinc cations in solution, as well sequestering zinc from fluorescent zinc–FluoZin-3 complexes, forming metal–chelation complexes. In addition, polyphenols were also tested as zinc-carriers across a liposome membrane, and not all compounds were observed to be zinc ionophores. The results from the comparison of both chelating strength capacity and ionophore activity are presented in Fig. 5, highlighting some interesting

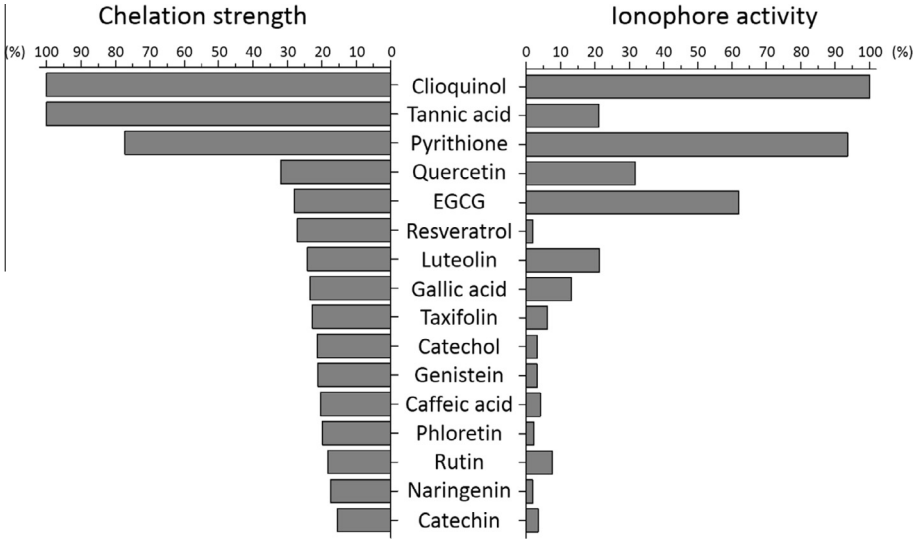

Fig. 5. Schematic comparison between zinc chelating strength and ionophore activity displayed by the different phenolic compounds, clioquinol and pyrithione.

observations. Generally, the compounds with a higher chelating capacity, also presented a high ionophore activity. However, some of the polyphenols did not follow this general trend. It is important to note that all the ionophore compounds could chelate the molecule, but not all the chelators have the ability to act as ionophores. The physicochemical properties of each of the polyphenols will directly have an effect on its ionophore activity, consequently defining the compound as metal sequestering agent or ionophore agent. However a deeper study is still needed to fully understand their mechanism of action.

Although the results from the liposomal assay presented herein demonstrate an active transport mechanism of zinc by the phenolic compounds, further investigation of their biological action *in vivo* needs to be done, since this was demonstrated in a controlled *in vitro* system and may not correlate to the conditions occurring in a living organism. In the present work, the rationale of chosen the tested concentrations of polyphenols and zinc to underline their biological effect at physiological levels was supported by our past studies where polyphenols and zinc were able to act modulating some of the central genes involved in zinc homeostasis (MT and ZnT1 genes), as well as increase the amount of cytoplasmic labile zinc, both *in vitro* using Hepa1-6 (mouse cell line) and HepG2 (human cell line), and *in vivo* using C57BL/6J mice (Quesada et al., 2007, 2011). In addition, it has been reported in 2007 that several pathways can be activated with only a few nanomolar concentrations of polyphenols or their metabolites (Schroeter & Bahia, 2007; Vauzour et al., 2007), emphasizing that even very few concentration of polyphenols could be enough to exert a biological effect, and, as reported by Kim et al., even those metabolites can maintain their ability to chelate and form complexes with metal ions.

4. Conclusions

It is confirmed that most of the natural occurring phenolic compounds used in this study have the ability to directly interact and form new structures (complexes) bound with zinc. In many cases these complexes act in a similar way to the control ionophores. We have reported a liposome assay that can be used as a tool for the rapid, high-throughput screening of families of polyphenols. In addition, this liposome system can be used to screen the ionophore activity towards other biometals of importance, such as Ca, Fe or Mg ions among others. Zinc-specific FluoZin-3 loaded liposomes were used to screen the zinc-ionophore activity of a selected library consisting of the most relevant dietary polyphenols, classified according to their zinc-ionophore strength capacity and their chelation efficiency, giving us a better knowledge of the importance of the structural conformation versus biological activity. Synthetic ionophore molecules are currently being used as potential drugs against several chronic diseases including Alzheimer's and different types of cancer, and as demonstrated, one of the mechanisms by which polyphenols exert their beneficial activity is by acting as zinc ionophores. Polyphenol–zinc ion complexes are yet to be investigated and more extensive studies are needed in order to elucidate their possible clinical potential.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.11.057.

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