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Compared Chemical Activity and Effects on Cell Viability of Various Antioxidant Compounds

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Abstract: ***Background:*** Oxidative stress plays a major role in the development of several diseases. A healthy food diet, supplying the organism with antioxidants, is generally believed to help prevent this stress.

Methods: With the aim of screening several antioxidants commonly found in food, a method using β pancreatic cells and based on the reduction of a tetrazolium sodium salt into colored formazan in the presence of antioxidant compounds was used. In parallel, the same compounds were investigated using a chromatographic online antioxidant detection system (Coads) based on the detection of the reduced form of the radical cation 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) which is first allowed to react online with the eluting compounds.

Results: The obtained data suggested that most phenolic compounds possessed both antioxidant and pro-oxidant properties. Gallic acid, in particular, showed among the highest antioxidant activities with the Coads method (322±46 µM Trolox equivalent), but caused a decrease in cell viability (-25%).

Conclusion: Moreover, when screening natural compounds for their antioxidant activity, combining both chemical and biological methods is a more targeted approach compared with the single use of either method.

Keywords: Oxidative stress, bioactive compounds, antioxidant, pro-oxidant, β pancreatic cells, ABTS.

1. INTRODUCTION

With thousands of chemically diverse compounds, food is one of the most complex natural matrices. Among these compounds, some are known for their bioactivity and positive effects on the human health [1]. Isolating, identifying and quantifying these often trace-amount molecules have therefore proved to be challenging tasks for chemical analysts.

The search for bioactive molecules in complex matrices, such as food, is typically a multistep procedure which involves chemical extractions using solvents of different polarity and selectivity classes, micro-fractionation on a solid support (SPE, preparative or semi-preparative chromatography, flash chromatography...), HPLC separations leading to pure compounds, structural determinations (FTIR, HR-MS, HR-NMR, 2D-NMR,...), and biological assays at almost each step of this process to determine the bioactivity of the sample of interest.

The number of fractions that need to be biologically tested increases during the course of the purification and can be overwhelming, so that choices are often made to focus efforts on particular extracts and discard others. Most of the time, the molecule of interest cannot be isolated because it is simply overlooked, for instance by not being detected during the fractionation (absence of chromophores with UV-Vis detection, absence of ionizable groups with mass detection, etc...). Besides, the active fraction may consist of a mixture of more or less active compounds, which prohibits the determination of the active molecule. Also, very fragile compounds can be degraded during long purification procedures. It appears therefore that bio-guided fractionation, an expensive, time-consuming and complicated endeavour, is also far from being a targeted one [1].

Among the wide range of biological assays, those directed at antioxidant compounds in relation to oxidative stress are certainly the most widely used. Oxidative stress is in fact generally accepted as a major participant in the development and progression of several diseases such as cardiovascular diseases, cancer and diabetes [2, 3]. It generates free radicals and also non radical species [4, 5]. Free radicals are highly reactive chemical species that can cause damage to cellular proteins, membrane lipids and nucleic acids, as well as impair the endogenous antioxidant defense systems [6]. The antioxidant systems involve both enzymatic and non enzymatic strategies. Enzymatic antioxidants include

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superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase. The β pancreatic cells have been used to study antioxidant response towards oxidative stress. Indeed, these cells express low activity of free radical detoxifying enzymes and redox-regulating enzymes such as catalase, superoxide dismutase and glutathione peroxidase [7-9].

As for non-enzymatic systems, common antioxidants are mostly provided by a healthy food diet and include vitamins A, C, and E, glutathione, α-lipoic acid, mixed carotenoids, coenzyme Q10 (CoQ10), minerals (copper, zinc, manganese and selenium), and phenolic compounds ((+)-Catechin, (-)-Epicatechin, Epigallocatechin gallate, Chlorogenic acid...) [10]. Antioxidant polyphenolic compounds, that exert both anti-inflammatory and antioxidant actions, protect β cells from oxidative stress [11].

With the aim of isolating the most effective bioactive phenolic compounds from natural sources, several assays have been developed to chemically measure the activity of antioxidants [12]. In recent studies, a Chromatographic Online Antioxidant Detection System (Coads) has been used to screen compounds in beer extracts and directly assess their antioxidant activity [13, 14]. This system separated compounds using reverse phase HPLC and after elution from the column they were submitted to two UV-visible detections: one for the phenolic compounds; and the other for the reduced form of the radical cation 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) after the compounds were allowed to react online with it. The most important antioxidant contribution (approximately 70% of the total antioxidant activity) came from prodelphinidin B3, procyanidin B3, catechin, ferulic and sinapic acids, as well as an unidentified compound. Also, the phenolic standards tested did not show equal antioxidant activities; gallic acid, with the best antioxidant response, was approximately 75 times more potent than ferulic acid, the least active compound.

Using chemical assays in the assessment of the antioxidant activity during multistep extraction and purification procedures can be highly advantageous by allowing instantaneous and easy testing of fractions of interest, faster analysis, and a more targeted fractionation process. Yet, until today, the chemical determination of the antioxidant activity of phenolic compounds remains entirely disconnected from the biological one, and there are no reported comparative assessments of the two techniques. This is in fact of the highest importance when it comes to evaluate the relevance of the chemical determinations as to what really occurs within a cell.

In this study, a cytokine-induced β-cell damage system was used to screen standard antioxidant compounds commonly found in food with the aim of selecting the best candidates for the prevention of the β-cell damage. The previously developed chemical Coads method was assessed comparatively with this biological system.

2. MATERIAL ET METHODS

2.1. Chemicals and Products

2,2'-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 4-hydroxy-3-methoxycinnamic acid (ferulic acid), 3,4-dihydroxycinnamic acid (caffeic acid), 3,4,5-trihydroxybenzoic acid (gallic acid), 3,4-dihydroxybenzoic acid (protocatechuic acid), chlorogenic acid, sinapic acid, catechin, epicatechin, epigallocatechin gallate (EGCG), ascorbic acid (vitamin C) and hydrogen peroxide (H_2O_2), freshly prepared from a 33% commercial solution, were purchased from Sigma-Aldrich (Seelze, Germany). B2 procyanidin (PB2) was purchased from Extra synthese society (Genay, France). All chemicals and solvents used were HPLC-grade and were purchased from VWR (Strasbourg, France). Ultrapure water was produced by a Synergy UV purification system (Millipore, Molsheim, France).

2.2. Cell Line

RINm5F rat insulinoma cell line (American Type Culture Collection, Manassas, USA) was obtained from β RIN-m cells that produced and secreted insulin. The supplemented culture medium (SCM) used consisted of Roswell Park Memorial Institute medium (RPMI-1640) with 10% of foetal bovine serum (Sigma) and 1% of an antimycotic solution (ABAM, Gibco®, Invitrogen, Grand Island, USA) containing 10 0000 U/mL of penecilline G, 10 mg/mL of streptomycin and 25 µg/mL of amphotericin B. Cells were grown at 37°C in a humidified atmosphere with 5% CO_2, and were trypsinized at 80% confluence using 0.05% trypsin EDTA (Sigma). SCM was renewed every 48 h.

2.3. Antioxidants Effect on Cells Viability

Viability assay was performed using CellTiter 96® AQueous One Solution Cell Proliferation Assay from Promega Corporation (Madison, USA). This colorimetric method is based on the reduction of a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], called MTS, into coloured formazan by the dehydrogenases of metabolically active cells. The amount of generated formazan is directly proportional to the number of living cells.

Briefly, cells were incubated for 48 h in 96-well treated microplates (BD Falcon™, Franklin Lakes, USA) at ca. 30,000 cells/200 µL of SCM. Antioxidants were then added to the cells at various concentrations (1, 5, 10, 20, 50, 100, 200, 300, 400 and 500 µg/mL) and allowed to act for 1 h. Alternatively, hydrogen peroxide (H_2O_2), an oxidative stress agent, was added after the 1-h incubation. Freshly prepared H_2O_2 (33%, Sigma) was diluted in SCM at 40µM and contact with the cells was allowed for 30 min as published previously [15].

After treatment, cells were washed in PBS and 100 µL of SCM containing 20 µL of MTS were added in each well. Plates were incubated for 2 h at 37°C, under 5% CO_2, and absorbance was measured at 490 nm in a Metertech 960 microplate reader (Metertech Inc., Taipei, Taiwan). Results were expressed in viability percentages compared to control.

2.4. Chromatographic Separation and Identification of Antioxidant Compounds

The conditions and equipment used for Coads method were as previously described [14]. Briefly, separation was carried out using a Waters HPLC system (Waters, Saint-Quentin-Fallavier, France), a Hypersil BDS C18 HPLC col-

umn (5 µm, 250 x 4.6 mm i.d., Thermo Scientific, Gometz-le-Châtel, France), and a mobile phase consisting of a water (0.1% formic acid, v/v)/methanol gradient delivered at 1 mL/min. The following gradient was used: 0-25 min, 3-25% B; 25-26 min, 25-18% B; 26-29 min, 18% B; 29-47 min, 18-30% B; 47-57 min 30% B; 57-67 min, 30-65% B; 67-77 min, 65% B. Detection was done at 254 nm for all studied compounds and at 412 nm for their respective antioxidant activities after post-column reaction with $ABTS^{\bullet+}$prepared as previously described [14]. Briefly, a stable stock solution of $ABTS^{\bullet+}$ was produced by mixing a 7 $mmol.L^{-1}$ aqueous solution of ABTS with a 2.5 $mmol.L^{-1}$ solution of potassium persulfate (final concentration) and allowing the mixture to stand in the dark at 4° C overnight. Before use, an $ABTS^{\bullet+}$ working solution was obtained by diluting the stock solution in ethanol to reach an absorbance of 0.70 (± 0.02) AU at 734 nm. The $ABTS^{\bullet+}$ solution was delivered at 0.5 mL/min.

Calibration graphs for each compound were drawn from data of three replicate injections of 20 µL of methanol/water (50/50, v/v) solutions of standard mixtures. Calibration curves (six data points, n = 3) were linear with R^2 values higher than 0.99. Each phenolic compound was quantified according to its corresponding authentic standard, while the antioxidant potential was determined as the concentration of Trolox required to produce an equivalent peak area and expressed as Trolox equivalent (µM).

2.5. Statistical Analysis

Data were analyzed by ANOVA (at a significance level of 95%) using Statgraphics Plus software. All samples were analyzed intriplicate.

3. RESULTS

3.1. Compounds and Oxidative Stress

The antioxidant activity of compounds was studied using a biological method based on the β pancreatic cells viability, with and without H_2O_2-induced oxidative stress.

With the aim of assessing the likely toxicity of the investigated compounds, they were added to β cells in the absence of H_2O_2 from 1 to 500µg/mL. Results showed that with eight out of the twelve molecules tested, no significant ($p>0.05$) toxic effects were observed, even at the highest amounts tested (Table **1**). As example, the case of catechin has been detailed. With catechin, the observed 1to 23% loss of viability was not significant ($p>0.05$), nor were the differences between the tested concentrations (Fig. **1a**). It is also noteworthy that with non toxic compounds, no significant increases in viability were observed either. The remaining four compounds lead to a noticeable loss of cell viability, but at concentrations that varied depending on the molecule considered. Sinapic acid, caffeic acid, and vitamin C showed toxicity at 200 µg/mL, 400µg/mL and 200µg/mL, respectively, while gallic acid showed higher cell toxicity, with a loss of viability at 5 µg/mL (Table **1**).

In a second set of experiments, the capacity of the compounds to prevent the loss of cells viability induced by oxidative stress have been tested. H_2O_2 was used as the source of the reactive oxygen species and a single stress treatment was applied [16, 17]. H_2O_2 was added at 40 µM to β pancreatic cells with and without initial antioxidant pretreatment. With untreated cells, viability decreased significantly in the presence of H_2O_2 down to between 48±8% and 13±6% com-

Table1. Effect of the investigated compounds on the viability of RINm5F β cells with and without oxidative stress induced by H_2O_2 at 40µM(n=3). Various concentrations were tested and a toxic concentration was determined in the absence of H_2O_2-induced stress, as well as antioxidant concentrations in the presence of H_2O_2-induced stress and the range of % of viability associated.

Compounds	Toxic concentration	Antioxidant concentration	Viability of H_2O_2–stressed cells (%) (data at antioxidant concentrations)		
	µg/mL (µM)	µg/mL (µM)	control	pretreated	
Catechin	nt	1-500 (3-2939)	34 ± 7	72 ± 22	65 ± 7
Sinapic acid	200 (892)	1-100 (4-446)	36 ± 3	58 ± 3	81 ± 9
Procyanidin B2	nt	5-500 (9-864)	48 ± 8	62 ± 9	52 ± 15
Vitamin C	200 (1136)	400-500 (2273-2841)	27 ± 6	56 ± 16	71,5 ± 22
Epicatechin	nt	10-200 (34-689)	35 ± 3	41 ± 9	48 ± 6
Ferulic acid	nt	10-20 (51-103)	35 ± 9	51 ± 5	50 ± 6
Caffeic acid	400 (2220)	50-300 (278-1665)	16 ± 3	33 ± 11	103 ± 17
Trolox	nt	100-500 (400-1998)	13 ± 6	26 ± 3	68 ± 2
Protocatechuic acid	nt	100-500 (649-3244)	36 ± 11	56 ± 6	56 ± 8
EGCG	nt	50-500 (109-1091)	32 ± 4	52 ± 6	140 ± 23
Gallic acid	5 (29)	na	37±2	33 ± 2	
Chlorogenic acid	nt	20-500 (56-1411)	15±4	38 ± 14	54 ± 7

na, no antioxidant effect. nt, no toxic effect.

paratively with unstressed control cells (Table **1**). When cells were initially incubated in the presence of antioxidant compounds prior to H_2O_2 addition, cell viability was significantly improved ($p>0.05$) with 11 out of the 12 molecules tested. Only the highly toxic gallic acid did not show antioxidant activity with respective cell viabilities of 33±2 % (at 500 µg/mL) up from 37±2% for the H_2O_2 control.

Active molecules showed significant improvement in cell viability at concentrations which varied considerably ranging from 1 µg/ml for catechin to 400 µg/mL for vitamin C (Table **1**). The strongest antioxidant effect was in fact obtained with a catechin pretreatment which resulted, at only 1 µg/mL, in a cell viability of 72±22% up from 34±7% for the H_2O_2 control. Increasing the concentration of the antioxidant generally did not result in significant increases in cell viability, as can be seen with catechin, where viability ranged from 72±22% at 1 µg/mL to 65±7% at 500 µg/mL (Fig **1b**/Table **1**). Lower concentrations seemed in fact to yield better results, which is of course particularly obvious with compounds that showed toxicity at higher concentrations. Such was the case with sinapic and caffeic acids, which yielded respective increases in cell viability of 1.6 and 2.1 times at 1 µg/mL and 50 µg/mL (Table **1**).

3.2. Comparative Assessment of the Antioxidant Activity Using the Biological and the Coads Methods

Solutions of eleven antioxidant compounds at equimolar concentrations (150 µM) (below toxicity in the test of the viability of RINm5F β cells (Table **1**)) were used to chemically determine the corresponding antioxidant activities using the Coads method, as well as to measure the increases in β cells viability following an H_2O_2-induced stress. The Coads method is meant to screen for compounds with antioxidant activity in a more direct and rapid fashion. Following chromatographic separation, compounds of an extract were mixed on-line with a stabilized solution of the $ABTS^{\bullet+}$radical, which was directed to a UV-vis detector. The presence of antioxidants, acting as radical scavengers, results in a reaction with $ABTS^{\bullet+}$ and a subsequent decrease in absorption detected as a negative peak at 412 nm. Results showed that the Coads-measured activities, expressed as trolox equivalents, varied considerably depending on the

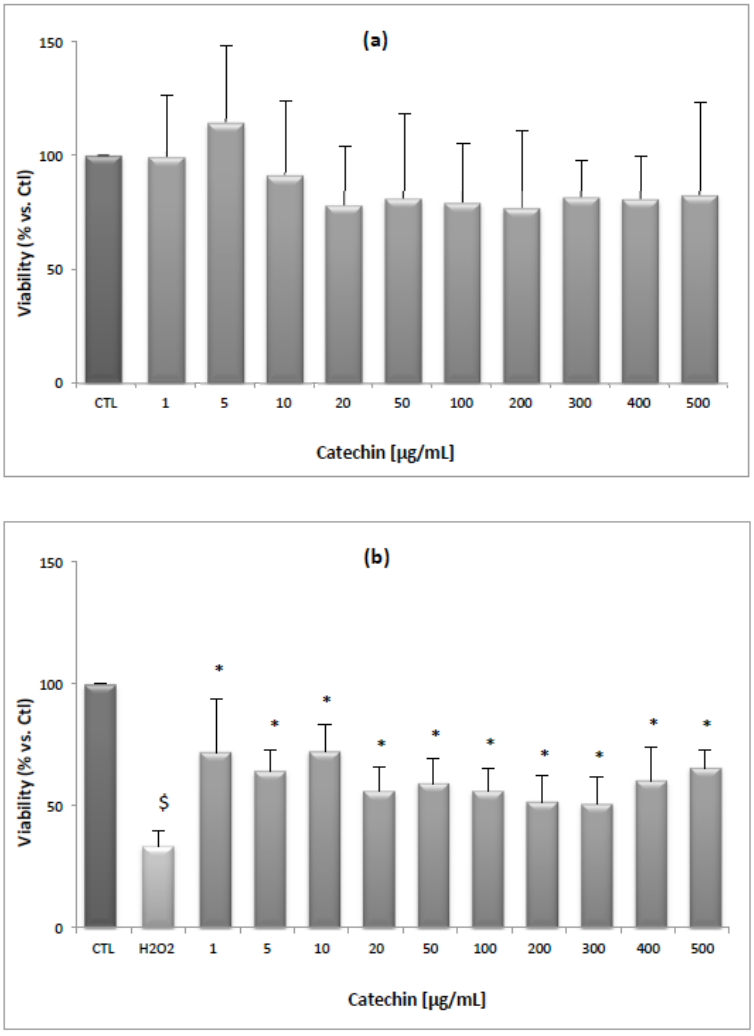

Fig. (1). Preventive effect of catechin on oxidative stress-induced lost viability.
(**a**) Effects of catechin on RINm5f cells viability. Results are expressed as mean ± SEM of 3 different experiments. (**b**) Effects of catechin on H_2O_2-induced lost viability. Results are expressed as mean ± SEM of 6 different experiments. $ represent significant difference between the control (Ctl) and stress (H_2O_2) and * between stress and catechin + stress. Medium was used as a control (Ctl).

Table 2. Antioxidant activity of the investigated compounds at 150 µM as determined by the Coads method (n=3). Activity is expressed as Trolox equivalent (µM).

Antioxidant compounds	Trolox equivalent (µM)
Gallic acid	322 ± 46
Procyanidin B2	279 ± 40
EGCG	255 ± 36
Caffeic acid	226 ± 32
Chlorogenic acid	205 ± 29
Epicatechin	201 ± 29
Vitamin C	190 ± 27
Sinapic acid	188 ± 27
Catechin	169 ± 24
Protocatechuic acid	62 ± 9
Ferulic acid	9 ± 4

molecule considered (Table **2**). With an activity of 322±46 µM Trolox equivalent, gallic acid proved to be the most active compound, ferulic acid was by far the least active one (9±4 µM Trolox equivalent), while other phenolic compounds such as chlorogenic acid and epicatechin showed average activities (Table **2**). As far as the biological data is concerned, it was EGCG which proved to be the most effective with a 2.8 times increase in cell viability, while ferulic acid displayed average activity (Table **3**). In fact, when comparing the biological and the chemical data, only four compounds seemed to show consistency in their measured activities with the two methods (EGCG, caffeic acid, vitamin C, catechin and protocatechuic acid). Almost opposite results between the two methods were obtained with sinapic acid, procyanidin B2 and gallic acid. This was particularly striking for gallic acid which showed among the highest antioxidant activities with the Coads method (322±46 µM Trolox equivalent), but caused a decrease in cell viability (Table **3**). With the remaining compounds (epicatechin, ferulic and chlorogenic acids) results were more or less different between the two methods.

4. DISCUSSION

The antioxidant compounds investigated in this study resulted in a significant increase in the viability β pancreatic cells following H_2O_2-induced stress. The effective concentration varied however considerably depending on the molecule considered, and some molecules did not show any positive *in vitro* effect. At high concentrations, some molecules, such as caffeic and sinapic acids and vitamin C, even induced a significant loss of viability. A toxic concentration could also be determined, which revealed that antioxidant molecules could show a deleterious effect, probably pro-oxidant. Similarly, the toxic concentration depended on the molecule considered, and the observed effect on β cells is apparently the result of combined antioxidant and toxic activities. It is therefore conceivable that with molecules which are highly antioxidant and toxic at the same time, the resulting biological effect might be negligible. This seems to be the case with gallic acid, which proved to be the most toxic molecule for β cells tested, and whose observed antioxidant effects were the weakest. Yet, when tested for antioxidant activity using the Coads method, they were the most active molecule. It can therefore be suggested that, in the cellular setting, the antioxidant and, probably, the pro-oxidant activities would cancel each other.

Table 3. Effect of the antioxidant activity of the investigated compounds at 150 µM on the viability of RINm5F β cells subjected to a stress by H2O2 at 40 µM (n=3).

Antioxidant compounds	Viability of H_2O_2-stressed cells (%)	
	Control	Pretreated
EGCG	32 ± 4	92 ± 9
Caffeic acid	16 ± 3	56 ± 12
Sinapic acid	36 ± 3	73 ± 13
Epicatechin	35 ± 3	63 ± 6
Chlorogenic acid	15 ± 4	43 ± 7
Catechin	34 ± 7	59 ± 10
Procyanidin B2	48 ± 8	68 ± 12
Ferulic acid	35 ± 9	50 ± 6
Vitamin C	27 ± 6	39 ± 5
Protocatechuic acid	36 ± 11	38 ± 7
Gallic acid	37 ± 2	12 ± 3

Reports show that, during their preparation and storage at room temperature, foods rich in phenolic compounds, such as cocoa, green tea and grape seeds, generate significant amounts of H_2O_2 responsible for pro-oxidant effects [18-22]. Among the phenolic compounds involved, there is caffeic acid in coffee and EGCG in tea [21, 22]. The pro-oxidant effects often involved reductions of metals such as Cu^{2+} and Fe^{3+} to Cu^{+} and Fe^{2+}, respectively, which can stimulate oxidative damage under certain assay conditions [23]. Zheng *et al*. have shown that hydroxycinnamic acids such as caffeic, chlorogenic and sinapic acids had a strong pro-oxidant activity due to Cu^{2+}chelation by their hydroxyl groups [24]. H_2O_2 production, responsible for the pro-oxidant activity and subsequent molecule toxicity, might be directly related to phenolic compound structures. Only OH-substituted at the 2 and 4 position of phenol yielded H_2O_2 and only o- and p-phenolic compounds undergo autoxidation [25]. Hydroxyl groups were involved in the oxidation of phenolic compounds into corresponding quinones, generating reactive oxygen species such as H_2O_2, in the presence of oxygen and metal ion transitions [25].

Obviously, the chemical method used on isolated compounds seems to report a one-sided story and certainly does not reflect the multiple activities molecules might have in a natural setting. Chemical evaluation of antioxidant activities would therefore have to be conducted using different methods and radicals, and in different oxidation conditions, as previously suggested [26]. Despite some consistency in our study between the chemical and the biological data, it seems that the two sets of data cannot be simply equated with the aim of translating what happens within a single reaction into actual biological activity. This would only be valid if antioxidant molecules were involved in single biochemical pathways, which is obviously not the case. As shown by Frankel and Meyer, several specific methods should be used to obtain chemical information that can be related directly to oxidative deterioration of food and biological systems [27]. Most of these molecules are in fact likely to show both antioxidant and pro-oxidant properties, concentration being a major player in the expression of one or other of the effects, and possibly other parameters such as the chemical structure, the test system used and the substrate considered [23].

With phenolic compounds, the pro-oxidant effects are reported at higher concentrations than the antioxidant ones. Yet, in our case toxic effects on β cells were observed with vitamin C at a concentration two times lower than the antioxidant concentration (Table **1**). It has in fact been previously shown for vitamin C and gallic acid, with protective effects against H_2O_2 at 4.0 mM and H_2O_2 induction with oxidative stress at 4 to 240 µM [28].

Conflicting results are also reported depending on the test used for activity measurement. For instance, while in our case chlorogenic acid showed neither antioxidant nor pro-oxidant activity with biological method, it has been reported to have antioxidant activity by DPPH radical scavenging test [29] and pro-oxidant activity by mediate DNA damage in the presence of Cu(II) [24]. This shows a need for improvement and harmonization of the tests. The use of more physiologically relevant radicals, such as hydrogen peroxide or superoxide anion radical, rather than ABTS which are not found naturally in the human body, has been suggested [30]. Also, cell viability assays, which are commonly used to study antioxidant compounds, are not designed to specifically measure the antioxidant activity. Wolfe *et al*. developed the Cellular Antioxidant Activity assay which used the ability of peroxyl radicals, reactive products of lipid oxidation, to induce the formation of a fluorescent oxidative stress indicator in the cell culture and measured the prevention of oxidation by antioxidants [31].

An antioxidant is a substance which, at low concentrations compared with those of oxidizable substrate, prevents or delays significantly substrate oxidation; a pro-oxidant is a substance that can cause oxidative damage to lipids, proteins and nucleic acids [30, 32]. Yet, things are not this simple, and the complexity of the biological environment, which is probably at the origin of many of the conflicting reports, is also the reason why the fate of phenolic compounds and their antioxidant and pro-oxidant activities in the human body have not yet been deciphered. For instance, pro-oxidant effects can also be beneficial because, by imposing a mild degree of oxidative stress, the levels of antioxidant defences might be raised, leading to overall protection [33]. In fact, our cells have to maintain a certain redox homeostasis. So, a healthy food diet combined with a dietary supplement, with compounds with antioxidant and pro-oxidant activity, could be a good recipe for oxidative stress prevention in the case of diabetes.

The food matrix is another complicating factor, since the complex and multiple interactions that is harbours are not represented by isolated compounds. Food antioxidants may interact either with each other leading synergistic and antagonistic effects, or with other food components leading to the enhancement or the attenuation of their activity [34].

5. CONCLUSION

It appears finally that the complexity of the natural environments cannot be reduced to a single reaction, such as $ABTS^{\bullet+}$radical reduction, or even to a β pancreatic cell. However, the Coads method, as well as other chemical methods, even when based on a single reaction, are highly valuable in detecting and quickly identifying antioxidant compounds in complex mixtures without the lengthy bioguided fractionation process. Applying this method to food would identify the antioxidant compounds, known or unknown, in a single chromatographic run. However, chromatographic method used combined active molecules should anyway have their activity confirmed by biological methods, especially when pro-oxidant activities are involved. Direct correspondence cannot be drawn between chemical reactions and *in vitro* biological assays, nor between the latter and what occurs within a living body. Yet, by giving indications as to the potential activity, chemical and biological assays remain necessary steps in the quest for antioxidant molecules.

ABBREVIATIONS

ABTS = 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)

EGCG = Epigallocatechin gallate

Coads = Chromatographic Online Antioxidant Detection System

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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Declared none.

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