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Antioxidant potential of aqueous plant extracts assessed by the cellular antioxidant activity assay

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Abstract

Several teas and herbal infusions are asserted as health promoters due to their content in antioxidant compounds. However the chemical methods of analysis to assess the antioxidants content do not give information regarding the biological activity of the studied substances. In the present study eight medicinal plants (3 *C. Sinensis, R. Canina, Menta Piperita, S. Officinalis, I. Paraguariensis* and *C. Arabica, C. Robusta blend*) as well as various antioxidant compounds naturally contained in these plants were screened to their antioxidant capacities, through chemical and cellular assays. Such medicinal plants were selected due to their wide utilization, both as infusions and as ingredients in cosmetics products and in food formulations. The main purpose was to assess their biological antioxidative capability measured in human cells, to assess their efficiency of protection against peroxyl radicals under physiological conditions, and later predict their *in vivo* activity. The plant extracts and antioxidant compounds here tested were found to be successfully absorbed, at different proportions, into human cells. However a lack of correlation between the chemical assay and the cell-based assay was observed. Care should be taken when assessing the antioxidant power with purely chemical methods. Unlike chemical assays, cell-based assays better reflect the complexity of in vivo models, considering some important aspects of uptake, cellular distribution and metabolism of the antioxidants in a cellular environment, and thus could sustain the research on antioxidants prior to animal studies or clinical trials.

Keywords

Cellular Antioxidant Activity, Epithelial Cells, Medicinal Plants, ORAC, Plant Extracts

1. Introduction

In the present study eight medicinal plants that are widely used as social beverages and/or traditional medicine were analyzed against the antioxidant activity of their aqueous extracts. The effect on free radical scavenging power was considered for extracts from *Camelia Sinensis* (green, black and white teas), *Coffea* (arabica coffee and robusta coffee blend), *Ilex paraguariensis* (yerba mate), *Salvia officinalis* (sage) and *Menta piperita* (peppermint) whole leaves as well as *Rosa canina* (hip rose) fruit.

Unfermented teas (green and white) as well as highly fermented tea (black) are widely consumed beverages obtained from the *C. sinensis*. Several literature data indicates that teas made from *C. sinensis* plant are beneficial to health and that many constituents have health promoting effects (Gupta *et al.*, 2008; Sharma and Rao, 2009). Green tea has been regarded to possess several health promoting properties (reviewed in Chacko *et al.*, 2010; Suzuki and Miyoshi, 2012), including oxidative stress protection, anti-cancer, anti-obesity, anti-atherosclerotic, anti-diabetic, anti-bacterial, and anti-viral effects (Brown, 1999; Friedman, 2007; Gawlik and Czajka, 2007; Pardo de Santayana *et al.*, 2005; Zhao *et al.*, 1989).

After water and tea, coffee ranks third in the list of most consumed beverages in the world. Coffee is rich in antioxidants (Wang and Ho, 2009), among them in chlorogenic acid. It has been reported a correlation between the consumption of coffee and the reduction of type-2 diabetes prevalence as well as the potential of altering gut flora and digestion (Tunnicliffe, 2008).

Yerba mate is widely consumed in South America as a social beverage (mate). Mate has been consumed for centuries for native people as social and medicinal beverage. Health promoting effects associated with mate include hypocholesterolemic, hepatoprotective and diuretic properties as well as CNS stimulant (reviewed in Heck and de Mejia, 2007). Research on the effects of Ilex paraguariensis in health and disease has confirmed its antioxidant (Lobato *et al.*, 2009; VanderJagt *et al.*, 2002), anti-inflammatory, anti-mutagenic and lipid-lowering activities (Bracesco *et al.*, 2011; Gao *et al.*, 2013).

Several health benefits were attributed to *Salvia officinalis*. Among these, anti-tumoral, antibacterial and anti-inflammatory properties have been reported (González *et al.*, 1987; Vladimir-Knežević *et al.*, 2014).

Peppermint tea, brewed from *Mentha piperita* leaves, is a commonly consumed tisane. Several potential health benefits have been reported for peppermint tea (reviewed in McKay and Blumberg, 2006), the organic extracts of the phytochemicals from leaves have a strong *in vitro* antibacterial activity against a range of pathogenic bacteria (Bupesh *et al.*, 2007; Sharafi *et al.*, 2010) as well as a strong antioxidative potential. The phenolic constituents of the leaves comprise rosmarinic acid and flavonoids.

For various preparations of rose hips and seeds, antioxidative and anti-inflammatory effects have been demonstrated; also some data was obtained in patients suffering from osteoarthritis treated with a powder of the seeds and husks of a *R. canina* subspecies (Chrubasik *et al.*, 2008). *Rosa canina L.* fruits were reported to have a high content on ascorbic acid, phenolics and flavonoids (Roman *et al.*, 2013).

These medicinal plants are primarily consumed in a "tea" form or tisane, made by steeping the dry leaves (or other plant parts) in hot water. Additionally, several cosmetics and nutritional products contain parts and/or extracts of these plants since their putative health promoting and antioxidant properties.

Even though some of the beneficial effects of these infusions may be attributable to antioxidants contained in these plants, to date very little information has been obtained on the antioxidant potential of different infusions that consider the physiological conditions, cellular uptake, distribution or metabolism issues. Therefore, the aims of the present study was to correlate the chemical antioxidant effect of these extracts with respect to their biological antioxidative capability measured inside human cells, to explore which product can trigger the highest increase of defense against peroxyl radicals and to later predict their *in vivo* activity.

To do so, the cellular antioxidant activity assay (CAA) described by Wolfe and Liu (2007) was used. This innovative cell-based method is useful to screen the effect of phytochemicals and to monitor the activity of reactive oxygen species (ROS) within a cell in a standard cell culture environment (Figure 1). CAA measures the ability of compounds to prevent the formation of a fluorescent probe, which is trapped within the cell cytosol, by peroxyl radicals generated by thermolysis of an exogenous oxidative stressor. The fluorescence intensity is proportional to the intracellular ROS levels and can be measured against the fluorescence inhibition of a Quercetin standard. The CAA assay is more biological relevant when compared with a pure chemical assay, since the cellular environment accounts some important aspects of bioavailability and physiological conditions, as well as efficacy of the antioxidants in a whole cell.

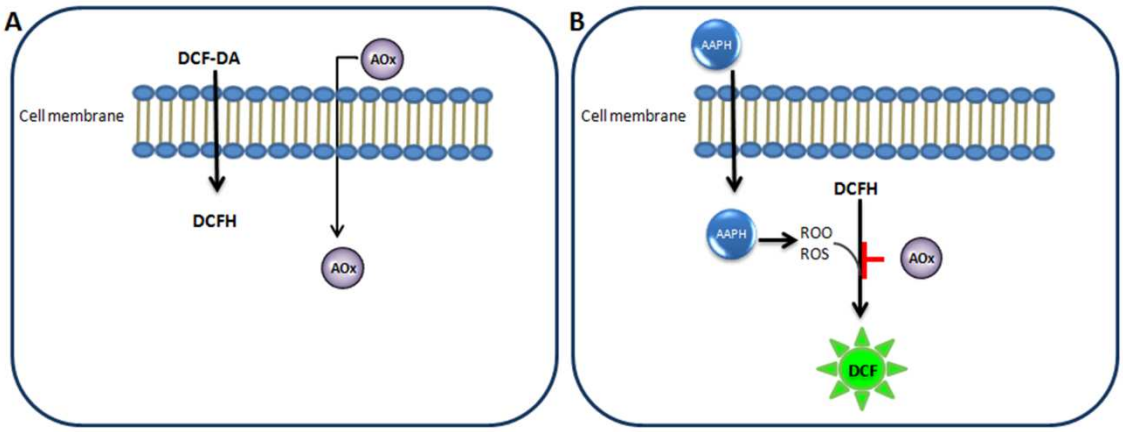

Figure 1. *Schematic representation of the CAA assay. A) HaCat cells were treated with the selected compounds (AOx) and the DCF-DA molecule that is taken up by cells by passive diffusion and deacetylated by cellular esterases to non-fluorescent DCFH which is trapped within the cells. B) DCFH is rapidly oxidized by free radicals, thus the addition of AAPH, a peroxyl radical initiator, leads to the oxidation of DCFH into DCF that emits fluorescence proportional to the level of oxidation. Under these conditions, a decrement in fluorescence signal in comparison to non treated cells indicates an antioxidant efficacy of the tested compound, since the antioxidants react with the peroxyl radicals preventing the formation of fluorescent DCF.*

To our knowledge the present study is the first to use a human cell line from epithelial origin to investigate the intracellular antioxidant activity. The skin is a major target of oxidative stress due to ROS that originate in the environment and in the skin itself. One of the most attractive reasons to conduct the study in keratinocytes is that the skin can be reached by chemical agents through both, topic application (making the skin the major target of cosmetics industry) as well as carried by the blood (making skin to one of the preferred targets of nutritional supplement and functional food industry). Furthermore, well established skin cell models can be experimented on without major ethics restrictions.

2. Materials and Methods

2.1. Samples preparation

C. sinensis teas, yerba mate and coffee were purchased at local supermarkets. Sage, hip rose and peppermint were purchased at Bonn local pharmacy (Germany). A stock solution was prepared fresh in each case by infusion with 100°C distilled water, at [5gr/L] final concentration, allowed to stand for 10 minutes stirring every 5 minutes. Of the resulting infusions 1 ml was filtered and diluted immediately before use. In addition, table 1 lists some main phytochemicals of these botanicals that were subjected to the same screening methods.

Table 1. *General information and description of main phytochemicals analyzed.*

Antioxidant	Present in:	Classification	Note
EGCg	white and green teas	flavanol	High CAA value in HepG2 cells(*)
Rutin	yerba mate, *C. sinensis*	flavonol glycoside	No effect on CAA in HepG2 cells(*)
Ascorbic Acid	rose hips	vitamin	No effect on CAA in HepG2 cells(*)
Caffeic Acid	coffee, green tea, white tea, mate	hydroxy-cinnamic acid	
Rosmarinic Acid	sage, mint	hydroxy-cinnamic acid	
Gallic Acid	*C. sinensis*	hydroxy-benzoic acid	
Quercetin dihydrate	yerba mate, *C. sinensis*	flavonol	Highest CAA value in HepG2 cells(*); CAA standard
Trolox	--	vitamin analog	ORAC standard
Ellagic acid	green tea, white tea	hydroxyl-benzoic acid	

*: From Wolfe and collaborators (2007, 2008).

2.2. Standards and Reagents

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and quercetin dehydrate standards, DMSO, ethanol, methanol, potassium phosphate, potassium hydroxide, fluorescein sodium salt, 2'-7'-dichlorodihydrofluorescein diacetate (DCF-DA), 2,2'-Azobis [2-methylpropionamide] dihydrochloride (AAPH) were purchased from Sigma-Aldrich (Italy). Ascorbic acid was obtained from Fagron Italia (Italy). Caffeic acid, rutin hydrate and rosmarinic acid were obtained from Sigma (Germany), gallic acid monohydrate was obtained from Hasenmüller (Germany) and epigallocatechin gallate (EGCg) was purchased from Sunphenon TAIYO (Germany).

Dulbecco's Modified Eagle's Medium (DMEM), Trypsin-EDTA solution 10X, trypan blue solution, glutamin penicillin streptomycin 10X solution were from culture grade and purchased from Sigma-Aldrich (Italy). Fetal bovine serum (FBS), Dulbecco's phosphate buffered saline (PBS) without Mg^{2+} and Ca^{2+} and Hank's balanced salts solution (HBSS) were purchased from Euroclone SpA (Italy).

All other chemicals were from analytical grade and purchased from common sources.

2.3. Cell culture

HaCat cell line (Boukamp *et al.*, 1988) was obtained from IZSLER Brescia (cat. BSCL168). Cells were cultured in complete DMEM-high glucose supplemented with 10% FBS, 2mM L-Glutamin, 50 µg/mL Penicillin and 50 µg/mL Streptomycin and were maintained at 37°C under an atmosphere of 5% CO_2.

2.4. Preparation of stock solutions

Phosphate buffer solution (75 mM, pH 7.4) was prepared and conserved at +4°C. Fluorescein 2 µM stock solution was prepared in phosphate buffer and preserved at +4°C protected from light. Trolox 10 mM stock solution was prepared in phosphate buffer. A 20 mM solution of DCF-DA was prepared in methanol. For the CAA assay a 200 mM stock solution of AAPH was prepared in water. Quercetin dehydrate was resuspended in DMSO and subsequently diluted in complete high glucose DMEM.

If not indicated diversely, all the stock solutions were aliquoted and preserved -20°C until use. Aliquots were thawed once. All working solutions were prepared fresh immediately before use.

2.5. Determining Antioxidant Activities

The preliminary analyses were aimed to screening the chemical antioxidant capacity, assessed by the oxygen radical absorbance capacity (ORAC) assay (Cao *et al.*, 1993) using Trolox as standard, while the biological antioxidant properties were evaluated using the CAA assay

described by Wolfe and Liu (Wolfe and Liu, 2007) and Quercetin as standard.

2.5.1. ORAC assay

The ORAC assay was used to determine the antioxidant capacities of commercial infusions and pure phytochemicals. To this aim the stock solutions of the samples were dissolved in phosphate buffer 75 mM (pH 7.4), and added in duplicate wells of a 96 well black plate (Greiner bio-one, Germany) containing 10nM of fluorescein sodium salt. In each plate Trolox dilutions (12.5 to 200 µM) were used as reference standard and phosphate buffer as blank. The plate was immediately placed in the fluorescence reader (Fluostar Optima, BMG Labtech, Germany) preheated at 37°C and incubated for 30 minutes before adding 25µl of fresh prepared AAPH 240mM, with the help of the instrument's pump. At physiological temperature AAPH decomposes at $1.36x10^6$/s, producing at most $1x10^{12}$ radicals/mL/s (Elmann *et al.*, 2013). Fluorescence emission was measured 45 times every 90 seconds at constant physiological temperature, by a 520nm filter with excitation at 485nm. Data were analyzed with MARS 2.0 software (BMG Labtech, Germany). Independent experiments were performed at least in triplicate.

Pearson's test was used to assess the significance of calibration curves linearity and the correlation among different parameters. ORAC values are expressed as mean ± Standard Error of 3 independent experiments and as mmol of Trolox Equivalents (TE) per 100 gr of dry weight or mmol of TE per mmol pure compound, depending on the sample type.

2.5.2. CAA assay

The CAA assay was used to determine the antioxidant activity of commercial infusions in epithelial cells. HaCat cells were pretreated with increasing concentrations of the respective samples and DCF-DA, a cell permeable precursor dye. A schematic description of the method is represented in Figure 1. In brief, HaCat cells were seeded in a black 96-well plate with transparent bottom (Greiner bio-one, Germany) at a density of $6x10^4$ cells in 100 µl of complete growth medium per well, and then incubated for 24 hours at 37°C and 5% CO_2. Triplicate wells were treated with 100 µl of extracts at varying concentrations diluted in treatment medium (DMEM + L-Glut without FBS) along with 25µM of DCF-DA and incubated for an hour. After incubation cells were washed with 100 µl of sterile PBS to remove the compounds not incorporated. The plate was immediately placed in the fluorescence reader (Fluostar Optima, BMG Labtec, Germany). Then 600µM AAPH was added to cells in 100µl HBSS medium, with the instrument's pump. Fluorescence kinetics was measured at 37°C every 5 minutes during an hour (excitation 485nm and emission 540nm). The integrated area under the fluorescence curve (AUC) was calculated for each sample, standard and control. AUC of each sample were normalized against the AUC of the control using the following formula: $AUC_{net} = AUC_{sample}/AUC_{control.}$

In every assay Quercetin was used as a reference standard, each independent experiment was performed at least in triplicate. Each plate included a triplicate control wells containing cells treated with DCF-DA and the peroxyl radical initiator, and blanks wells containing cells treated with DCF-DA and HBSS buffer instead of AAPH stressor. Peripheral wells of the plate were not used because are known to show high variability in comparison with inner wells, these wells were filled with 100 µl of sterile PBS during incubation steps and fluorescence reading.

Raw data were analyzed with MARS 2.0 Optima Data Analysis software. Pearson's test was used to assess the significance of calibration curves linearity and the correlation among different parameters. Results are expressed as mean ± Standard Error for at least three individual experiments. Based on the quercetin antioxidant standard curve the quercetin equivalents (QE) values of unknown samples were calculated. CAA values are expressed as mmol of QE per 100 gr of dry compound or per mmol of pure compound, depending on the sample type.

2.6. Viability and Cytotoxicity

Cell viability was determined within a hemacytometer by trypan blue exclusion. While the cytotoxicity of each compound toward HaCat cells was assessed through the LDH cytotoxicity assay according to the manufacturer's instructions (Innoprot, Spain). This colorimetric assay is based on the cytosolic lactate dehydrogenase activity, which is released into the culture medium by broken cells and upon treatment with toxic concentrations of a sample substance. Free LDH concentrations were read spectrophotometrically. Hence, non cytotoxic concentrations of the samples were used in the CAA assay.

3. Results

Based on the dry weight of the used plants, the ORAC values (µmol TE/gr) indicated that between the infusions tested green tea possesses the highest radical quenching property (2412 ±169; n=4) against peroxyl radicals (figure 2A), followed by white tea (2242 ±207; n=3), peppermint (1438 ±366; n=3), sage (1351 ±722; n=3), yerba mate (1195 ±39; n=3), black tea (1064 ±128; n=4), coffee (906 ±10; n=3) and last by rose hips (330 ± 109; n=3). Furthermore, on comparing with ascorbic acid (1050 µmol TE/gr ±80; n=3) 5 out of 8 plant extracts showed a higher antioxidant capacity assessed by ORAC assay, black tea resulted nearly equivalent to Vitamin C, while an equal dry weight of coffee or hip rose showed lower ORAC values.

To further evaluate the antioxidant capacity elicited *in vitro* the CAA assay was assessed in a biological model characterized by HaCat human keratinocytes. The reference standard, Quercetin, exhibited a dose-dependent inhibition of AAPH induced fluorescence in HaCat cells at

concentrations ranging from 10 to 1µM, as depicted from the curves showed in figure 3. The results indicate that all the plant infusions were effectively absorbed into cells, although at different rates. As illustrated in figure 2B, the intracellular antioxidant activity (µmol QE/gr dry weight) of green tea resulted the highest (170.4 ±5, n=3) followed by white tea (81 ±2, n=3) and yerba mate (46.5 ±3.3, n=3). Sage tea (35.3 ±3.9, n=3) and peppermint (27.9 ±1.6, n=3) resulted fourth and fifth by CAA assay, respectively. Black tea (22.7 ±1.5, n=3), coffee (11.7 ±1.3, n=3) and rose hips infusion (2.9 ±0.3, n=3) showed limited antioxidant capacities when compared to the other infusions, ranking on the three last positions, through both CAA and ORAC methods.

To further investigate the oxidative stress response elicited by these botanical infusions, some pure phytochemical substances that are present in these medicinal plants were analyzed. The complete list of the tested substances is described in table 1. As showed in figure 2 (panels C and D) rosmarinic acid, a phenolic acid contained in sage and mint extracts, showed a lower CAA value (0.19 ±0.004 µmol QE/µmol, n=3) but the highest ORAC value (6.3 ±0.1 µmol TE/µmol, n=3). Of note, the unexpected high CAA value of ellagic acid, a hydroxybenzoic acid contained in green tea, that have a much higher intracellular antioxidant activity against peroxyl radicals than all the compounds tested (1.24 ±0.2 µmol QE/µmol, n=3). However, ellagic acid showed a intermediate antioxidant activity assessed with the ORAC test (2.12 ±0.5 µmol TE/µmol, n=3) ranking at the sixth position once compared with the other phytochemicals. Clearly Quercetin, a flavonoid contained in green, white and black teas as well as in yerba mate, showed a high antioxidant activity in the CAA assay, ranking second and followed by EGCg (0.5 ±0.1 µmol QE/µmol, n=3), the most abundant catechin reported in green tea and white teas but that is not present in yerba mate and black tea. Gallic acid, caffeic acid, rutin and ascorbic acid showed a slight cellular antioxidant activity.

Using regression analyses, the relationship between CAA and ORAC values were determined. Under our experimental conditions and among the aqueous extracts the ORAC values were weakly correlated to CAA values (R^2=0.76, n=8). Nevertheless, no significant correlation was observed with pure phytochemicals (R^2=0.016, n= 9).

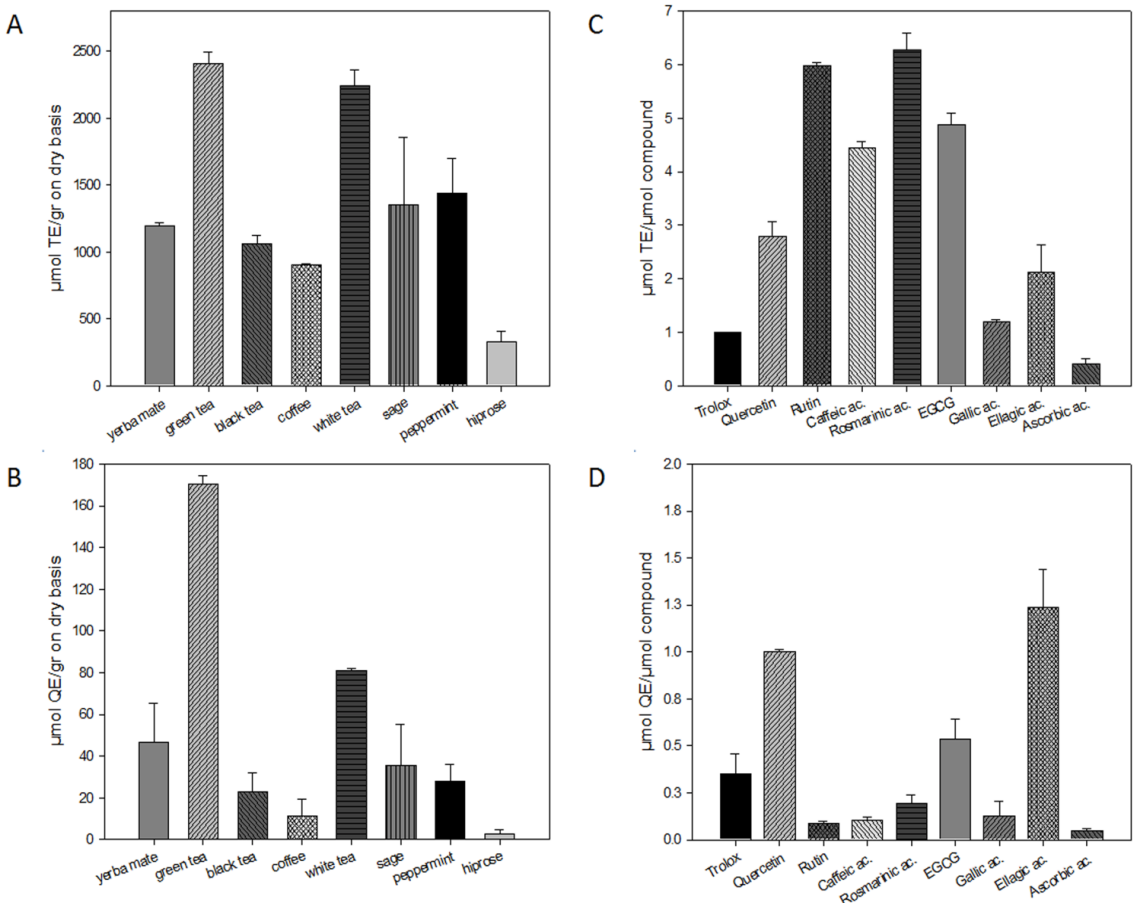

Figure 2. *Antioxidant activity of medicinal plants and phytochemicals against peroxyl radicals. Panels A) and B) represent the ORAC and CAA assays, respectively, determined in water extracts from I. paraguariensis (yerba mate), C. sinensis (green, black and white teas), C. arabica and C. robusta blend (coffee), S. officinalis (sage), Menta piperita (peppermint) and R. canina (hip rose). Panels C) and D) represent the ORAC and CAA values, respectively, determined in the main phytochemicals analyzed.*

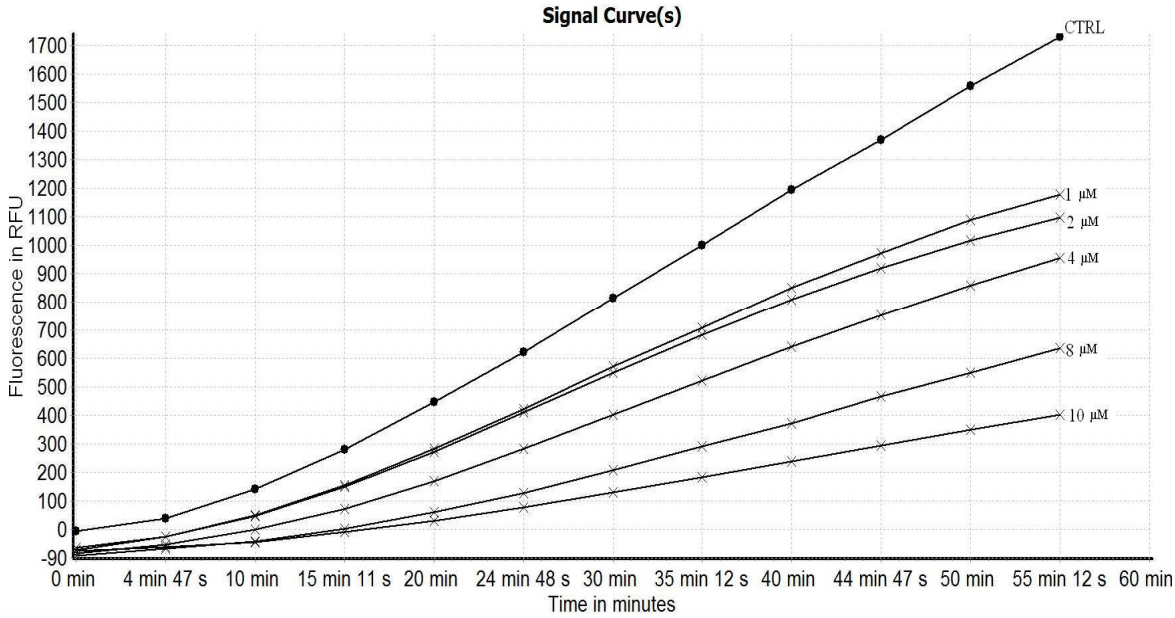

Figure 3. *Cellular Antioxidant Activity of Quercetin dehydrate in HaCat cells. The graph represents a typical curve of inhibition of oxidation over time by a quercetin standard. Cells were pretreated for an hour with DCFH-DA and a Quercetin standard (1 to 10 µM). After addition of AAPH the fluorescence intensity was read for an hour. Quercetin inhibited the increment of fluorescence in a dose dependent manner. CTRL: non treated cells.*

4. Conclusion

The present study reports the screening of 8 medicinal plant extracts and its main phytochemicals for their antioxidant activity using 2 different approaches: cell based (CAA) and non-cell based (ORAC) assays. A rather wide variation of antioxidant power was observed between the different extracts and pure compounds through both methods.

Among the infusions analyzed and under our experimental conditions, green tea extracts showed the maximum inhibitory effect from oxidation with the highest CAA and ORAC values, followed by white tea. The last had an ORAC nearly as high as green tea but showed only half CAA value. However, care should be taken because several parameters are expected to determine the concentration of polyphenols that can be extracted from an infusion. Among them, but not only, are the sample heterogeneity, the variety of the plant, the degree of milling and the blending (Wang and Ho, 2009; Barreira *et al.*, 2013; Leung *et al.*, 2001).

Our results showed that there is a lack of correlation between the intracellular and the chemical-based antioxidant activities, these findings match with other reports confirming that a high ORAC value does not at all guarantee for a high CAA value, and *vice versa* (Olsen *et al.*, 2013; Wolfe and Liu, 2008). Chemical methods, like ORAC, evaluate the potential of a sample in counteracting an oxidant molecule. Even today the chemical methods remain an important tool in the early stage of the screening. However, unlike chemical methods, the cellular models better reflects the physiological conditions, taking into consideration not only the cellular uptake and distribution but the scavenging effect of the antioxidant molecules in a cellular environment, as well as the upregulation of the antioxidative enzymes, their transcription and the modulation of the redox cell signaling, and thus could sustain the research on antioxidants prior to animal studies or clinical trials (Lopez-Alarcon and Denicola, 2013; Wolfe and Liu, 2007).

The lack of correlation between antioxidant activities of plant extracts and pure phytochemicals can be explained in part with the synergic effect that is expected to be obtained with a complex matrix rich in phytochemicals in comparison with pure substances, due to the heterogeneity in compounds and their content.

As a final point, the present report shows the potential of HaCat keratinocytes cell line as a model to measure the intracellular antioxidant capacity of phytochemicals and natural extracts that could be functional for the cosmetic and nutritional supplements formulations. This cellular model enables to study some key aspects to usefulness of the potential antioxidants *in vivo*, thus making this cell model a suitable intermediate step between the chemical methods and the expensive studies *in vivo.*

Abbreviation list

AAPH: 2,2'-Azobis [2-methylpropionamide] dihydrochloride
AUC: area under the fluorescence curve
CAA: cellular antioxidant activity
CNS: central nervous system
DCF-DA: 2'-7'-dichlorodihydrofluorescein diacetate
DMEM: Dulbecco's modified eagle's medium
DMSO: dimethyl sulfoxide
EDTA: ethylenediaminetetraacetic acid
EGCg: epigallocatechin gallate

FBS: fetal bovine serum
HBSS: Hank's balanced salts solution
LDH: lactate dehydrogenase
ORAC: oxygen radical absorbance capacity
PBS: phosphate buffered saline
QE: quercetin equivalents
ROS: reactive oxygen species
TE: trolox equivalents

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