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An in vitro comparative study of the antioxidant activity and SIRT1 modulation of natural compounds

Jonathan Fusi[a,1], Sara Bianchi[b,*,1], Simona Daniele[c], Silvia Pellegrini[a], Claudia Martini[c], Fabio Galetta[a], Luca Giovannini[b], Ferdinando Franzoni[a]

[a] Department of Clinical and Experimental Medicine, University of Pisa, Italy
[b] Department of Translational Research and New Technologies in Medicine and Surgery, Pharmacology, Medical School, University of Pisa, Italy
[c] Department of Pharmacy, University of Pisa, Italy

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ABSTRACT

Oxidative stress arises from an imbalance between the production of free radicals and antioxidant defences. Several studies have suggested that dietary antioxidants (such as polyphenols and berberine) may counteract oxidative stress through the involvement of the Sirtuin 1/Adenosine Monophosphate-Activated Protein Kinase (SIRT1/AMPK) pathway. The aim of this study was to evaluate the direct and specific antioxidant activity of some natural compounds, as well as their ability to modulate the expression of SIRT1 and the activation of AMPK.

Quercetin, tyrosol, ferulic acid, catechin, berberine and curcumin were evaluated for their specific and direct antioxidant activity with TOSC assay. Their ability to modulate SIRT1 and AMPK was assessed by immunoblotting assay, while their cytotoxicity by CellTiter-Blue Cell Viability Assay.

No statistically significant decrease ($p > 0.05$) in the number of viable cells was found upon challenging with the natural compounds. Quercetin exhibited the highest antioxidant activity against peroxyl radical and peroxinitrate derivates, while curcumin showed the best anti-hydroxyl activity with respect to the other compounds and, most importantly, respect to the reference antioxidants. Finally, all the tested compounds significantly increased the SIRT1 expression and the activation of AMPK.

Our results clearly disclose the specific antioxidant activity of these natural compounds and their ability to increase SIRT1 expression and AMPK activation.

1. Introduction

Oxidative stress is commonly defined as an imbalance between the production of reactive oxygen species (ROS, free radicals) and antioxidant defences [1,2]. Reactive oxygen and nitrogen species (RONS) are ubiquitous reactive derivatives of O2 and nitrogen metabolism, responsible for numerous types of cell damage. At this purpose, there is a general agreement on the fact that a chronic imbalance between formation of RONS and antioxidant systems is a relevant determinant involved in the pathogenesis and development of a variety of chronic and degenerative diseases, including aging, cancer, cardiovascular disease and neurodegenerative disorders (Alzheimer's and Parkinson's Diseases) [2–5]. In support of this view, there has been growing evidence that oxidative stress and specific human diseases can be prevented by including in the diet plant foods that contain large amounts of antioxidants, such as vitamins C, E or natural antioxidants, such as polyphenols [6,7]. Dietary antioxidants act as free radical scavengers, radical chain reaction inhibitors, metal chelators, oxidative enzyme inhibitors and antioxidant enzyme cofactors [8–11].

Nowadays, a huge amount of studies support the beneficial role of antioxidants to counteract RONS both in sedentary subjects and in athletes. In this respect, many supplements, characterized by natural

Abbreviations: AMPK, adenosine monophosphate-activated protein kinase; APAB, 2,2′-azo-bisamidinopropane; BSA, bovine serum albumin; CCL_4, carbon tetrachloride; DMEM, Dulbecco's modified eagle's medium; DMSO, dimethyl sulfoxide; DTPA, diethylenetriaminepentaacetic acid; FBS, fetal bovine serum; GSH, reduced glutathione; GSSG, oxidised glutathione; HRP, horse radish protein; KMBA, α-cheto-γ-(methylthiol)butyric acid; mTOR, mechanistic target of rapamycin; NAD^+, nicotinamide adenine dinucleotide; NAMPT, nicotinamide phosphoribosyl transferase; PBS, phosphate buffered saline; PMSF, phenyl-methane-sulfonyl-fluoride; PVDF, polyvinylidene difluoride; RONS, reactive oxygen and nitrogen species; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SIN-1, 3-morpholinosydnonimine *N*-ethylcarbamide; SIRT1, sirtuin 1; TOSC, total oxidant scavenging capacity; T-TBS, tween-tris buffered saline

* Corresponding author at: Via Roma 55, 56126, Pisa, Italy.
E-mail address: sara.bianchi1987@hotmail.it (S. Bianchi).
[1] These authors equally contributed to this work.

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compounds, are commercialized with the "therapeutic" purpose of improving the antioxidant defences. Some of these supplements are characterized by polyphenols, such as ferulic acid, quercetin, tyrosol, catechin and curcumin and natural compounds as berberine.

Polyphenols, such as ferulic acid, quercetin, tyrosol, catechin and curcumin, are secondary metabolites of plants and are usually classified based on their chemical structure, according to the number of phenol rings and on the basis of structural elements [12–14]. Research in animal and human models has shown that these compounds possess a wide range of biological protective effects, such as anti-inflammatory, antibacterial, anti-allergic and antioxidant ones. In addition, the protective role of polyphenols has been showed in cardiovascular, neurodegenerative and neoplastic diseases [15–17].

Berberine, a quaternary ammonium salt from the protoberberine group of benzylisoquinoline alkaloids, is the principal component for many popular medicinal plants (Coptidis chinensis, Phellodendron chinense and Mahonia bealei) [18]. The numerous pharmacological activities of berberine, in the last two decades, have been attracting high-level interests within the scientific community [19,20]. Indeed, berberine has been shown to have therapeutic effects on hypoglycemia, inflammation, cancer and it is also useful for prevention and treatment of Alzheimer's disease and cerebral ischemia [21,22]. Berberine is also able to ameliorate and alleviate oxidative stress both in vitro and in vivo models [23–26].

The fundamental mechanism of action underlying polyphenols' and berberine's impact on human health is probably represented by their action on Sirtuin 1 (SIRT1) and Adenosine Monophosphate-Activated Protein Kinase (AMPK), two important proteins involved in many pathophysiological processes, able to activate each other: AMPK activates SIRT1 (by increasing the Nicotinamide phosphoribosyltransferase (NAMPT)levels) and SIRT1 stimulates AMPK through LKB1 deacetylation [27–30].

SIRT1 is a NAD+ dependent histone/protein deacetylase able to deacetylate a lot of substrates, including p53, NF-kB, FOXO transcription factors, Ku-70, PPAR-γ, and PGC-1α, with roles in cellular processes ranging from energy metabolism to cell survival [31–36].

AMPK is a fuel-sensing enzyme activated by a decrease in a cell's energy state that inhibits anabolic processes and increases the catabolic ones with the aim of restoring ATP reserve [37]. Recent works suggest a relationship between SIRT1/AMPK and the oxidative stress, underlining how their activation could be crucial in this context and potentially protective against oxidative stress [38].

Based on this premise, the aim of this study was to evaluate the direct and specific antioxidant activity of some natural compounds (Fig. 1), such as quercetin, tyrosol, ferulic acid, catechin, berberine and curcumin. Moreover, to shed light on these compounds' molecular mechanisms, we evaluated the ability of these substances to increase the expression of SIRT1 and the activation of AMPK.

2. Materials and methods

2.1. Chemicals and antibodies

Ascorbic acid, KMBA (α-cheto-γ-(methylthiol)butyric acid)), 2,2′-azo-bisamidinopropane (ABAP), Diethylenetriaminepentaacetic acid (DTPA) and 3-morpholinosydnonimine N-ethylcarbamide (SIN-1) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell culture reagents were purchased from Lonza (Basel, Switzerland) and Gibco-BRL (Grand Island, NY). General laboratory chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Carlo Erba (Milano, Italy). Tyrosol, (+)- Catechin, Berberine Chloride Hydrate, Curcumin, Quercetin Dihidrate and Ferulic Acid also were from Sigma-Aldrich (St. Louis, MO, USA). All substances were dissolved in DMSO (Sigma-Aldrich, St. Louis, MO, USA). The final concentrations of DMSO in culture were between 0.05–0.2%, which had no effect on cell viability (data not shown).

Ripa Lysis Buffer System, Protease Inhibitor Cocktail, Phosphatase Inhibitor Cocktail C, phenyl-methane-sulfonyl-fluoride (PMSF), Sodium Orthovanadate and Sodium Pyrophosphate were from Santa Cruz Biotechnology (California, USA). CellTiter-Blue Cell Viability Assay was purchased from Promega (Madison, USA). Reagents, protein markers and membrane for Western Blot were from Biorad Laboratories (California, USA), Rabbit polyclonal antibody against SIRT1 (H-300, sc-15404), mouse monoclonal antibody against Actin (C-2, sc-8432), goat anti-rabbit IgG-HRP antibody (sc-2004) and goat anti-mouse IgG-HRP antibody (sc-2005) were purchased from Santa Cruz Biotechnology (California, USA). Rabbit polyclonal antibody against Phosho-AMPKα (Thr172, #2531) and rabbit polyclonal antibody against Total AMPKα (#2532) were from Cell Signaling Technologies (Massachussets, USA). Luminata Crescendo Western HRP Substrate for chemiluminescent detection of bands were from Millipore (Massachussets, USA). Glutathione Fluorometric Assay Kit (GSH, GSSG and Total) was purchased from BioVision (California, USA).

2.2. TOSC assay

The total oxidant scavenging capacity (TOSC) assay was described in detail in our previous work [39]. Briefly, peroxyl radicals were generated by thermal homolysis of 20 mM ABAP at 35 °C in 100 mM potassium phosphate buffer, pH 7.4. Hydroxyl radicals were generated at 35 °C by the iron plus ascorbate-driven Fenton reaction (1.8 μM Fe3+, 3.6 μM EDTA, and 180 μM ascorbic acid in 100 mM potassium phosphate buffer, pH 7.4). Peroxynitrite was generated from the decomposition of SIN-1 in the presence of 0.2 mM KMBA, 100 mM potassium phosphate buffer, pH 7.4, and 0.1 mM DTPA (Diethylene Triamine Penta Acetic Acid), at 35 °C. The concentration of SIN-1 was varied to achieve an ethylene yield equivalent to the iron–ascorbate and ABAP systems. Reactions with 0.2 mM KMBA were carried out in 10 ml vials sealed with gas-tight Mininert® valves (Supelco, Bellefonte, PA) in a final volume of 1 ml.

Ethylene production was measured by gas-chromatographic analysis of 200 μl aliquots taken from the head space of vials at timed intervals during the course of the reaction. Analyses were performed with a Hewlett-Packard gas chromatograph (HP 6890 Series, Andoven, MA) equipped with a Supelco DB-1 (30 × 0.32 × 0.25 mm) capillary column and a flame ionization detector (FID). The oven, injection and FID temperatures were respectively, 35, 160 and 220 °C. Hydrogen was the carrier gas (at a flow rate of 1 ml/min); a split ratio of 20:1 was used. Total ethylene formation was quantified from the area under the kinetic curves that best define the experimental points obtained for control reactions and after addition of quercetin, tyrosol, ferulic acid, catechin, and berberine and curcumin during the reaction. TOSC values were quantified from the equation $TOSC = 100 - (SA/CA \times 100)$, where SA and CA are the integrated areas for sample and control reaction, respectively.

TOSC values were quantified from the equation $TOSC = 100 - (SA/CA \times 100)$, where SA and CA are respectively the area under the curve (AUC) for sample and control reaction. A TOSC value of 0 corresponds to a sample with no scavenging capacity. A TOSC value of 100 is attributed to a compound that entirely suppresses the ethylene formation whereas a pro-oxidant compound shows a negative TOSC value. Consequently, antioxidants and pro-oxidants molecules can be distinguished by the obtained results. The linearity of dose–response curve quercetin, tyrosol, ferulic acid, catechin, and berberine and curcumin concentration (μM) and the antioxidant (TOSC value) response was tested and good correlation coefficients (generally greater than 0.9) were obtained at the different doses used to test the validity of our experiments (Fig. 2). Each experiment was performed in duplicate to account for the intrinsic variability of the method. The results obtained with quercetin, tyrosol, ferulic acid, catechin, and berberine and curcumin were expressed in TOSC units, and compared to each other. In our hands, the coefficient of variation (CV) of the