OxiCyan®, a phytocomplex of bilberry (*Vaccinium myrtillus*) and spirulina (*Spirulina platensis*), exerts both direct antioxidant activity and modulation of ARE/Nrf2 pathway in HepG2 cells

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ABSTRACT

The antioxidant activity of plant extracts requires compelling evidence of the physiological functions of anti-oxidants at the cellular level. In order to assess the antioxidant power of the phytocomplex OxiCyan®, a bilberry extract functionalized on spirulina, traditional chemical methods (DPPH and ABTS) were compared to *in vitro* cellular responses by using HepG2 human hepatocyte carcinoma 85011430 cell lines. OxiCyan® was found to act on HepG2 cells in a dual mode: with a direct effect on ROS scavenging and as a cytoprotective agent via induction of ARE/Nrf2 pathway. Direct ROS scavenging activity of OxiCyan® depended on the anthocyanin moiety provided by bilberry, whereas the gene activation of the ARE/Nrf2 pathway was triggered by spirulina chemical constituents. The comparative analysis of chemical and cellular assays indicated contrasting results. OxiCyan® showed a high antioxidant activity at the cellular level by both scavenging ROS and inducing the gene expression of natural antioxidant cell defense.

1. Introduction

The oxidative stress is caused by the unbalance in the prooxidant-antioxidant activity of living cells, with prooxidant activities leading to the production of Reactive Oxygen Species (ROS), which cause cell damage (Oldham & Bowen, 1998). ROS scavenging occurs naturally in living cells and includes both enzymatic and non-enzymatic mechanisms; however, when prooxidants prevail, these natural measures become insufficient. In this cases, uptake of antioxidant can be supported by the diet through intake of fruits, vegetables and dietary supplements (Prior, Cao, Prior, & Cao, 2000). The antioxidant capacity of foods and food supplements is often measured by chemical reaction of the plant extract or ingredient with ROS sources, including the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), peroxyl radical ($ROO^{\cdot}$), hydroxyl radical ($OH^{\cdot}$), singlet oxygen ($^1O_2$), and peroxynitrite ($ONOO^-$) (MacDonald-Wicks, Wood, & Garg, 2006). The most widely used methods are: oxygen radical absorbance capacity (ORAC), total antioxidant activity by using 1,1-diphenyl-2-picryl-hydrazil stable radical (DPPH˙), the ferric reducing antioxidant potential (FRAP), the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS˙⁺) assay, and many others (Cheli & Baldi, 2011). However, when the chemical antioxidant power of molecules and extracts tested with these methods is compared with a cellular-based activity, different and often contrasting results are obtained. This is because these traditional chemical tests do not inform about the physiological functions of antioxidants. These chemical assays have been discredited for years and strongly discouraged by the main health monitoring authorities. For instance, EFSA scientific substantiation of health claims on the protection of body cells and molecules from oxidative damage requires at least one appropriate marker of oxidative modification of the target molecule assessed *in vivo* (EFSA-NDA-Panel, 2017). In 2012 USDA's Nutrient Data Laboratory (NDL) removed the USDA ORAC Database for Selected Foods from the NDL website due to mounting evidence that the values indicating antioxidant capacity have no relevance to the effects

of specific bioactive compounds, including polyphenols, on human health (see https://bit.ly/2IQQxWt).

The study of antioxidant effects on live cell models currently represents the most advanced approach for demonstrating antioxidant physiological effects. In this work we evaluated the antioxidant activity of OxiCyan®, a phytocomplex composed by spirulina (*Spirulina platensis*) and bilberry (*Vaccinium myrtillus*) produced by Biosfered Srl (Italy) through a patented process. Antioxidant properties of spirulina are correlated to the presence of β-carotene, α-tocopherol and other components with free radical scavenging properties and antioxidant activity, including chlorophyll, phycoerythrin and phycocyanin (Abu Zaid, Hammad, & Sharaf, 2015; Fernandez-Rojas, Hernandez-Juarez, & Pedraza-Chaverri, 2014; Nouri & Abbasi, 2018). Bilberry antioxidant power is normally associated to the high radical scavenging activity of anthocyanins (Babova, Occhipinti, Capuzzo, & Maffei, 2016; Bornsek et al., 2012; Colak et al., 2017). We assessed both the chemical and cellular-based antioxidant capacity of OxiCyan®. Chemical assays were performed with the DPPH and ABTS assay, whereas for the cellular-based antioxidant activity, we used HepG2 human hepatocyte carcinoma 85011430 cell lines and the technology based on Light-Up Cell System (LUCS). The LUCS test is based on the production of cellular radical species following the addition in the culture medium of a photo-inducible fluorescent nucleic acid biosensor. The effect of light application in presence of the cellular biosensor triggers the production of singlet oxygen which in turn causes the production of ROS in a biochemical cascade linked to an increase of emitted fluorescence (Derick et al., 2017). Besides evaluating the direct antioxidant activity, we also performed, the ARE/Nrf2 live cell assay, which is a reporter gene approach that measures the ability of the sample to activate ARE (Antioxidant Response Element) DNA promoter sequence following the nuclear release of Nrf2 transcription factor from the Keap1/Nrf2 cytosolic complex. Upon exposure to oxidative stress or other ARE activators, Nrf2 is released from Keap1 and translocates to the nucleus, where it can bind to the ARE, leading to the expression of antioxidant and phase II enzymes that protect the cell from oxidative damage. This genomic pathway (also called "natural antioxidant cell defense") is well-known to increase cell capacity to adapt to oxidant stress or aggression (Ma, 2013) and the Nrf2-dependent luciferase reporter gene assay has been successfully used to revealed the activation of the expression of Nrf2-regulated gene by natural products (Odongo et al., 2017; Wu, Liu, Xu, Chen, & Lin, 2018). Moreover, reporter assays can be used as qPCR alternatives as they directly link the response element of interest (here ARE) to gene expression (here luciferase, mimicking expression of ARE induced genes such as *HO-1, GST, NQO1*).

Here we show that OxiCyan® exerts a strong antioxidant activity at the cellular level and that chemical antioxidant assays may lead to contrasting and sometimes misleading results.

2. Materials and methods

2.1. Plant material, cell lines, reagents and chemicals

OxiCyan® (batch number SVP01-1801001) and extracts of *Spirulina platensis* (Gomont) Geitler (CAS: 724424-92-4) and *Vaccinium myrtillus* L. (CAS: 84082-34-8; EINECS: 281-983-5) were kindly provided by Biosfered Srl (Italy). Certificate of analysis, technical sheets and materials safety data sheet of OxiCyan® are available from Biosfered upon request. The method of extraction and production of OxiCyan® are covered by the company trade secret, whereas bilberry extract was obtained from dried *V. myrtillus* berries by using ethanol and water (70:30 v:v) while spirulina extract was obtained by drying and milling cultivated *S. platensis*.

Dulbecco's Modified Eagle Medium (DMEM) was purchased from Thermo-Fisher (Waltham, MA USA), cell lysis solution and luciferin were purchased from BPS Bioscience (San Diego, CA, USA), HepG2 human hepatocyte carcinoma, 85011430 cell lines, Fetal Calf Serum (FCS), ethanol (95%), potassium ferricyanide (99.8%), 1,1-diphenyl-2-picryl-hydrazil stable radical (DPPH˙) (> 98%) and ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid) (98%) were purchased from Sigma-Aldrich (Milan, Italy).

2.2. Total phenolic compounds content

The total phenolic compounds content (TPC) of OxiCyan®, *S. platensis* and *V. myrtillus* was determined by the Folin-Ciocalteu's method (Singleton, Orthofer, & Lamuela-Raventos, 1999). Gallic acid (GA) was used for the preparation of the calibration curve and the results were expressed as mg GA g^{-1} d.wt. All measurements were repeated three times.

2.3. Total anthocyanin content

The total anthocyanin content (TAC) of OxiCyan®, *S. platensis* and *V. myrtillus* was measured using the differential pH method (Elisia, Hu, Popovich, & Kitts, 2007). Cyanidin chloride (CC) was used as standard and the total anthocyanin content was expressed as mg CC g^{-1} d.wt. All measurements were performed in triplicate.

2.4. Total phycobiliprotein content

The determination of the phycobiliprotein content of OxiCyan®, *S. platensis* and *V. myrtillus* was based on the Bennett and Bogorad's protocol (1973). Briefly, 100 mM phosphate buffer pH 7 was added to samples in a 1:10 (w/V) ratio. After vortexing, samples were ultra-sonicated for 30 min at room temperature and then centrifuged at 3500g for 10 min. The spectrophotometric quantification (mg ml^{-1}) of C-phycocyanin (PC), allophycocyanin (APC) and C-phycoerythrin (PE) was obtained according to follow equations:

$$PC = \frac{OD_{615} - 0.474(OD_{652})}{5.34}$$

$$APC = \frac{OD_{652} - 0.208(OD_{615})}{5.09}$$

$$PE = \frac{OD_{562} - 2.41\ PC - 0.849\ APC}{9.62}$$

2.5. HPLC-DAD-ESI-MS/MS analysis of of OxiCyan®, S. platensis and V. myrtillus anthocyanins

OxiCyan®, *S. platensis* and *V. myrtillus* extracts were analyzed for their anthocyanin content. The HPLC system consisted of an Agilent Technologies 1200 coupled to a diode array detector and a 6330 Series Ion Trap LC-MS System (Agilent Technologies, USA) equipped with an electrospray ionization source. The chromatographic separation was carried out at constant flow rate (0.2 ml min^{-1}). The column was a reverse phase C18 Luna column (3.00 μm, 150 × 3.0 mm i.d., Phenomenex, USA), maintained at 25 °C by an Agilent 1100 HPLC G1316A Column Compartment. The UV–VIS spectra were recorded between 220 and 650 nm and the chromatographic profiles were registered at 220, 280, 360 and 520 nm. Tandem mass spectrometry analyses were performed operating in positive mode. The nitrogen flow rate was set at 5.0 ml min^{-1} and maintained at 325 °C, whereas the capillary voltage was set at 1.5 kV. Helium was used as a collision gas. Compound identification was carried out by comparison of the retention time and UV–VIS/MS spectra with those of authentic reference compounds or using literature data. The binary solvent system was MilliQ H_2O acidified with 0.1% (v/v) formic acid (Solvent A) and MetOH 50% v/v acidified with 10% v/v formic acid (Sigma-Aldrich, USA) (Solvent B). The elution method involved a multistep linear solvent gradient changing from an initial concentration of 85% A and 15% B to 55% A and 45% B in 15 min. Finally, the gradient was 30% A and

70% B in 20 min. The concentration of solvent A was decreased to 2% and was maintained for 5 min before the next injection. Sample injection volume was 15 μl. No anthocyanins were detected in *S. platensis*.

2.6. Fatty acid analysis of OxiCyan®, V. myrtillus and S. platensis

Lipophilic extracts of OxiCyan®, *S. platensis* and *V. myrtillus* were obtained by Soxhlet extraction by using cyclohexane (1:10, w/v). After extraction, the solvent was removed with a nitrogen flow. The Soxhlet extract was esterified with boron tri-fluoride (10% w/v in methanol). Fifty μg heptadecanoic acid (C17:0) were added as internal standard (Maffei & Peracino, 1993). The fatty acid methyl esters (FAME) were obtained by acid catalysis according to Christie and Han (2010) and were dehydrated with anhydrous $MgSO_4$. FAME identification and quantification was performed by GC-MS (5975T, AgilentTechnologies, USA) and by GC-FID (GC-2010 Plus, SHIMADZU, Japan), respectively. The GC carrier gas was helium with a constant flux of $1\ ml\ min^{-1}$, and separation was obtained with a non-polar capillary column ZB5-MS (30 m length, 250 μm diameter and stationary phase thickness of 0,25 μm, 5% phenyl-arylene and 95% poly-dimethyl siloxane) (Phenomenex, USA). The following temperature conditions was used: injector 250 °C, oven initially at 60 °C, held for 1 min and raised to 180 °C ($10.0\ °C\ min^{-1}$ and held for 1 min). Then the temperature was brought to 230 °C ($1.0\ °C\ min^{-1}$ and held for 2 min) and to 320 °C ($15\ °C\ min^{-1}$) held for 5 min. Same column and chromatographic condition were used for both GC-MS and GC-FID analyses. MS parameters were: ionization energy of the ion source was set to 70 eV and the acquisition mode was set to 50–350 *m/z*. Compounds were identified through comparison of mass fragmentation spectra with reference NIST 98 spectra or by comparison of Kovats indexes and internal standard co-injection of pure standards (Sigma-Aldrich, USA). FAME quantification was obtained by internal standard. At least three technical replicates were run.

2.7. Chemical antioxidant capacity of OxiCyan®, S. platensis and V. myrtillus by ABTS and DPPH assays

The free radical scavenging activity of OxiCyan®, *S. platensis* and *V. myrtillus* was measured using 1,1-diphenyl-2-picryl-hydrazil stable radical (DPPH˙) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS˙⁺) assay, respectively based on the methods described by Vladimir-Knezevic et al. (2011) and by Re et al. (1999). Briefly, 0.1 mM solution of DPPH˙ in ethanol was prepared and this solution (1 ml) was added to sample solution in ethanol (3 ml) at different concentrations (0.63–80 μg/mL). The mixture was shaken vigorously and left to stand for 30 min in the dark, and the absorbance was then measured at 517 nm. ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation (ABTS˙⁺) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. ABTS˙⁺ solution was diluted with ethanol, pH 7.4, to an absorbance of 0.70 at 734 nm and equilibrated at 30 °C.

For each extract, the antioxidant activity (AA) of samples was calculated using the following equation:

$$AA\% = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100$$

where: A_{blank} is the absorbance of blank, and A_{sample} the absorbance of sample at 517 nm.

IC_{50} values were determined from the plot of scavenging activity against the compounds concentrations, which were defined as the total antioxidant necessary to decrease the initial radical concentration by 50%.

Table 1
Chemical composition of OxiCyan®, *Spirulina platensis* (Spirulina) and *Vaccinium myrtillus* (Bilberry) extracts. Values are expressed as mg g^{-1} d. wt. (± standard deviation). n.d. = not detectable (below the detection limit).

Specification	OxiCyan®	Spirulina	Bilberry
Phycocyanin	49.70[a] ± 1.97	256.12[b] ± 9.26	n.d.
Allophycocyanin	32.12[a] ± 1.74	44.12[b] ± 1.80	n.d.
Phycoerithrin	29.36[a] ± 1.27	21.79[b] ± 1.13	n.d.
Total Phycobiliproteins	111.18[a] ± 1.58	322.03[b] ± 3.85	n.d.
Total Anthocyanins	10.24[a] ± 0.85	n.d.	261.25[b] ± 10.94
Total Unsaturated Fatty Acids	14.39[a] ± 1.99	12.18[a] ± 0.28	2.49[b] ± 0.34
Total Saturated Fatty Acids	18.35[a] ± 0.58	20.00[a] ± 0.03	0.82[b] ± 0.04

In the same row, different letters indicate significant (P < 0.05) differences.

2.8. Antioxidant activity of OxiCyan®, S. platensis and V. myrtillus on human HepG2 cells using the Light-Up Cell System (LUCS) method

Samples of OxiCyan®, *S. platensis* and *V. myrtillus* were solubilized at a final concentration of $50\ mg\ ml^{-1}$ in DMEM culture medium. Solutions were then centrifuged at 8700 rpm for 10 min and experiments were performed with the supernatants. pH of samples was compatible with the assay. HepG2 cells were seeded in 96-well plates at a density of 75,000 cells/well in DMEM medium supplemented with Fetal Calf Serum (FCS) and kept in the incubator for 24 h at 37 °C in the presence of 5% CO_2. Cells were then incubated with samples (8 concentrations obtained by serial log2 dilutions) for 4 h at 37 °C in the presence of 5% CO_2. Experiments were done in DMEM medium without FCS. At least two independent experiments were performed each on triplicate wells. LUCS assay measures the ability of an antioxidant to neutralize oxidative stress and the effect is measured by a delay in the kinetic evolution of fluorescence emission according to Derick et al. (2017). Briefly, cells were treated after the 4 h incubation with the fluorescent biosensor during 1 h and fluorescence was measured (RFU at 535 nm) according to a recurrent 480 nm LED application procedure (20 iterations) of the whole 96-well plate. Kinetic profiles were recorded and dose-response curves were also calculated. The antioxidant cell index (AOP index) was calculated from normalized kinetic profiles according to the formula:

$$AOP\ \ index\ \ (\%) = 100 - 100 \left(\frac{\int_{20}^{0} RFU_{sample}}{\int_{20}^{0} RFU_{control}} \right)$$

Dose-response curves, obtained by compiling AOP indices according to Log of the sample concentration, were submitted to a sigmoid fit according to the formula:

$$AOP\ index = \frac{AOP\ index_{min} + (AOP\ index_{max} - AOP\ index_{min})}{1 + 10^{Log(EC_{50} - SC)HS}}$$

where SC = sample concentration, HS = Hill slope, EC_{50} (50% efficacy concentration). EC_{10} and EC_{90} were also evaluated.

2.9. Activation of the ARE/Nrf2 gene pathway by OxiCyan®, S. platensis and V. myrtillus in HepG2 cells

Samples of OxiCyan®, *S. platensis* and *V. myrtillus* were solubilized and ARE Reporter–HepG2 cells were seeded as described above. Cells were then incubated with samples (8 concentrations obtained by serial log_2 dilutions) for 17 h at 37 °C in the presence of 5% CO_2. The ARE Reporter – Hep G2 cell line contains a firefly luciferase gene under the control of ARE stably integrated into Hep G2 cells. This cell line is validated for the response to the stimulation of sulforaphane. Specifically, sulphoraphane was diluted into assay medium and 5 μl of dilution was added to each well. Cells are incubated at 37 °C in a CO_2 incubator for17 h. Luciferase assay was performed using the BPS-

Table 2

Anthocyanin composition of OxiCyan® and *Vaccinium myrtillus* (Bilberry) extract. Values are expressed as mg g^{-1} d. wt. (± standard deviation).

Compound	Molecular ion [M] + (m/z)	Daughter ion (m/z)	OxiCyan®	Bilberry
Delphinidin-3-O-galactoside	465	303	1.46[a] ± 0.04	32.20[b] ± 1.52
Delphinidin-3-O-glucoside	465	303	1.39[a] ± 0.04	35.55[b] ± 1.45
Delphinidin-3-O-arabinoside	435	303	0.92[a] ± 0.03	23.52[b] ± 0.96
Cyanidin-3-O-galactoside	449	287	1.28[a] ± 0.03	32.61[b] ± 1.31
Cyanidin-3-O-glucoside	449	287	0.97[a] ± 0.02	24.86[b] ± 1.00
Cyanidin-3-O-arabinoside	419	287	0.49[a] ± 0.01	12.54[b] ± 0.47
Petunidin-3-O-galactoside	479	317	1.57[a] ± 0.05	40.04[b] ± 1.73
Petunidin-3-O-glucoside	479	317	0.13[a] ± 0.00	3.27[b] ± 0.21
Petunidin-3-O-arabinoside	449	317	0.32[a] ± 0.01	8.06[b] ± 0.32
Peonidin-3-O-galactoside	463	301	0.64[a] ± 0.02	16.44[b] ± 0.74
Peonidin-3-O-glucoside	463	301	0.87[a] ± 0.02	22.11[b] ± 0.95
Peonidin-3-O-arabinoside	433	301	0.20[a] ± 0.01	5.06[b] ± 0.26

In the same row, different letters indicate significant (P < 0.05) differences.

Table 3

Fatty acid chemical composition of OxiCyan®, *Spirulina platensis* (Spirulina) and *Vaccinium myrtillus* (Bilberry) extracts. Values are expressed as mg g^{-1} d. wt. (± standard deviation), Ki = Kovats Index. n.d. = not detectable (below the detection limit).

Compound	Ki	OxiCyan®	Spirulina	Bilberry
Palmitoleic acid	1878	2.47[a] ± 0.17	2.03[a] ± 0.03	n.d.
Palmitic Acid	1886	17.89[a] ± 0.59	19.49[a] ± 0.02	0.72[b] ± 0.08
Linoleic acid	2082	6.31[a] ± 0.90	4.89[b] ± 0.16	1.22[c] ± 0.17
Oleic acid	2085	0.88[a] ± 0.05	0.91[a] ± 0.01	0.14[b] ± 0.01
Elaidic acid	2093	0.34[a] ± 0.02	0.12[b] ± 0.10	n.d.
Stearic Acid	2133	0.47[a] ± 0.01	0.51[a] ± 0.01	0.11[b] ± 0.03
γ-Linolenic acid	2220	4.37[a] ± 0.85	4.23[a] ± 0.18	1.12[b] ± 0.19

In the same row, different letters indicate significant (P < 0.05) differences.

Table 4

Chemical antioxidant activity of OxiCyan® as compared to *Spirulina platensis* (Spirulina) and *Vaccinium myrtillus* (Bilberry) extracts. Values are expressed as IC$_{50}$ (in mg ml^{-1}) (± standard deviation).

Assay	OxiCyan®	Spirulina	Bilberry
DPPH (IC$_{50}$)	64.20[a] ± 2.87	129.74[b] ± 3.67	1.43[c] ± 0.10
ABTS (IC$_{50}$)	20.03[a] ± 0.73	38.49[b] ± 1.40	0.17[c] ± 0.01

In the same row, different letters indicate significant (P < 0.05) differences.

Table 5

Antioxidant power of OxiCyan®, *Spirulina platensis* (Spirulina) and *Vaccinium myrtillus* (Bilberry) extracts in HepG2 cells by using the LUCS approach.

Specification	OxiCyan® (mg ml^{-1})	Spirulina	Bilberry (µg ml^{-1})
Highest antioxidant index	10.00	n.d.	780
EC$_{90}$	9.11	n.d.	400
EC$_{50}$	4.34	n.d.	190
EC$_{10}$	2.07	n.d.	90

Bioscience (San Diego, CA, USA) ONE-Step™ Luciferase Assay System by adding 100 µl of ONE-Step™ Luciferase Assay reagent per well at room temperature for 15 min and luminescence was read on a Varioskan equipment (ThermoFisher, Waltham, MA USA), according to a standard operating procedure designed by Anti Oxidant Power (AOP, France). Incubation with a mix (BPS Bioscience, USA) comprising cell lysis solution and luciferin (substrate of luciferase) for 30 min. Luminescence values (RLU) reveal luciferase gene expression following ARE promotion. Results are presented as Fold Increase (FI) with respect to the control values according to the formula:

$$Fold\ Increase\ (FI) = \frac{RLU_{sample}}{RLU_{control}}$$

Data are reported assuming min and max asymptotes by sigmoid fit, the dose response curve is then calculated. EC$_{10}$, EC$_{50}$, EC$_{90}$ values and Hill Slopes are calculated from the sigmoid fit according to the formula:

$$FI = FI_{min} + \frac{FI_{max} - FI_{min}}{1 + 10^{Log(EC_{50} - SC)HS}}$$

where SC = sample concentration and HS = Hill slope.

2.10. Statistical analysis

At least three biological replicates were always performed for chemical analyses. ANOVA followed by Tukey–Kramer's HSD post-hoc test (P < 0.05) was used to determine significant differences. A nested ANOVA was calculated for fluorimetric analyses. All statistical analyses were performed by using the SYSTAT 10 software.

3. Results and discussion

Because of the presence of dietary antioxidants, consumption of vegetables is known to reduce the risk of developing diseases correlated to the generation of ROS, including cardiovascular disease, degenerative or chronic diseases and cancer (Stanner, Hughes, Kelly, & Buttriss, 2004). In humans, antioxidant networks include both nonenzymatic and enzyme-assisted mechanism of ROS scavenging; however, most of the antioxidant capacity of plant foods and extract is evaluated with chemical methods, which do not provide evidence of cellular functionality (Derick et al., 2017). The results of this work show that the phytocomplex OxiCyan® exerts both chemical and cellular-based antioxidant activity by directly quenching the ROS and activating the natural cellular antioxidant responses.

OxiCyan® is characterized by the presence of the typical chemical constituents of spirulina and bilberry. A direct comparison of OxiCyan® with the plant extracts of spirulina and bilberry shows that spirulina contributes mainly to the phycobiliproteins and fatty acid content, whereas bilberry provides the anthocyanin moiety (Table 1). With regards anthocyanins, both OxiCyan® and bilberry extract were characterized by the presence of delphinidin, cyanidin, petunidin and peonidin glycosides (Table 2). Preliminary assays of spirulina extracts showed anthocyanin contents below the detection limit (data not shown). In both OxiCyan® and bilberry, petunidin 3-*O*-galactoside was the most abundant anthocyanin, followed by consistent amounts of delphinidin 3-*O*-galactoside, delphinidin 3-*O*-glucoside, and cyanidin 3-*O*-galactoside (Table 2). Supplementary Fig. S1 shows the typical HPLC profile of OxiCyan® anthocyanins. The chemical composition of the bilberry extract was is in line with the literature data (Babova et al., 2016; Benvenuti, Brighenti, & Pellati, 2018).

The lipid composition of OxiCyan® is characterized by the presence of both saturated and unsaturated fatty acids. Table 3 shows the direct comparison among OxiCyan®, spirulina and bilberry fatty acid profiles.
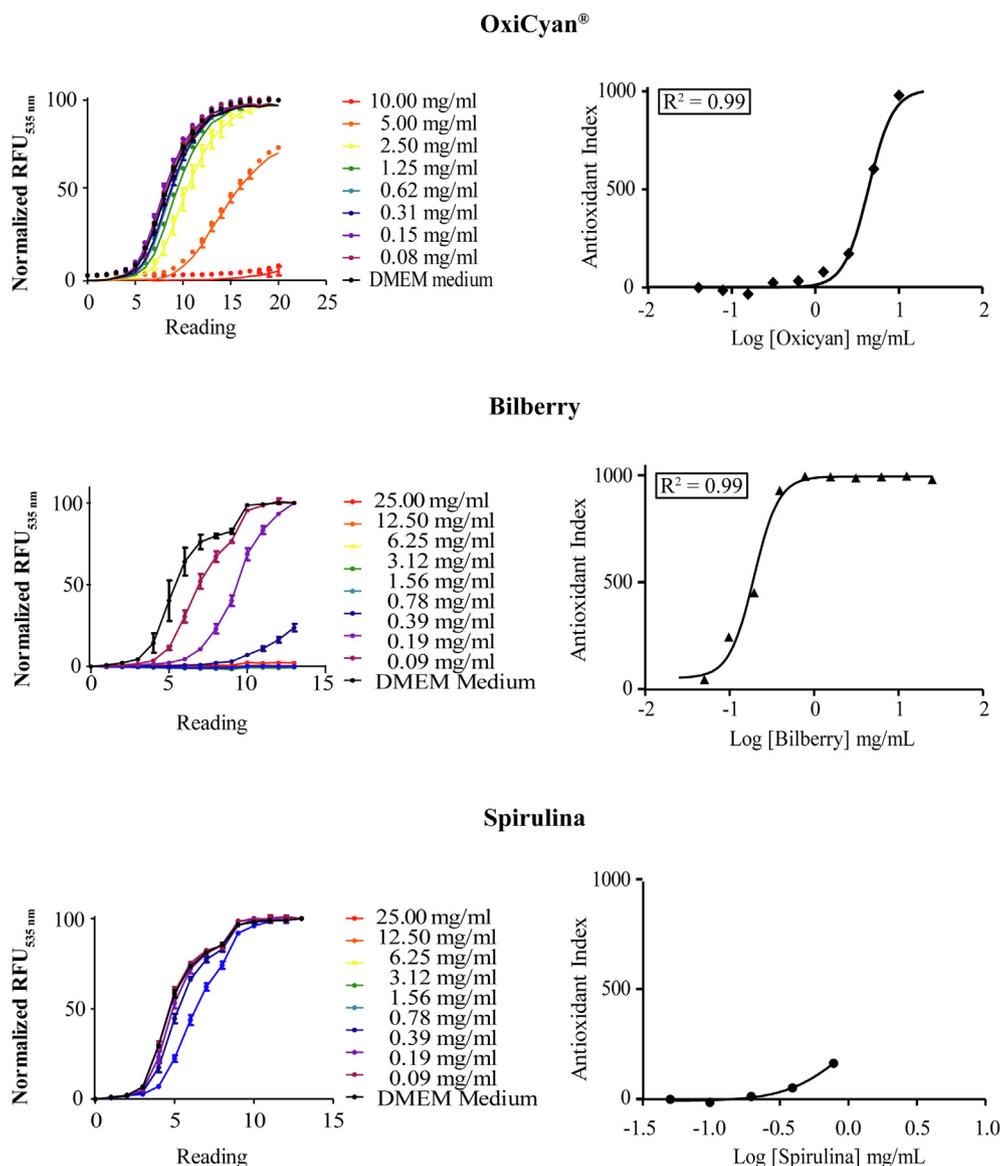
OxiCyan®

Bilberry

Spirulina

Fig. 1. Direct antioxidant activity of OxiCyan®, *Spirulina platensis* (Spirulina) and *Vaccinium myrtillus* (Bilberry) extracts on HepG2 cells. For each extract, the left panel shows the kinetic raw data, whereas the right panel shows the dose-response data. OxiCyan® shows a high antioxidant activity which is depending on the contribution of bilberry anthocyanins. Spirulina does not show any antioxidant activity. Bars indicate standard deviation.

The unsaturated fatty acid contribution to OxiCyan® is given by spirulina, in which these compounds are the most abundant fatty acids. The unsaturated fatty acids are represented by γ-linolenic acid (the most abundant polyunsaturated fatty acid in both spirulina and OxiCyan®), followed by linoleic and palmitoleic acids. Bilberry shows a low amount of both saturated and unsaturated fatty acids and was found to lack both palmitoleic acid and elaidic acid (Table 3). The chemical composition of spirulina and bilberry fatty acids was in line with the literature data on lipid composition (Gustinelli, Eliasson, Svelander, Alminger, & Ahrne, 2018; Ramadan, Asker, & Ibrahim, 2008; Wang et al., 2015).

Having assessed the chemical composition of OxiCyan®, we evaluated its chemical antioxidant capacity by assaying the free radical scavenging activities by ABTS and DPPH radical assays. A direct comparison was made with spirulina and bilberry extracts. In general, the extracts were more active as antioxidants when tested by the ABTS assay (Table 4), because the steric accessibility of DPPH nitrogen-centred radical strongly affects the reaction rate of antioxidant compounds (Babova et al., 2016; Prior, Wu, & Schaich, 2005). As expected, there was a wide variability among the methods used; however,

OxiCyan® showed a high antioxidant capacity that clearly depended on the bilberry contribution. In fact, the bilberry extract always showed the highest antioxidant capacity when compared to spirulina. The bilberry antioxidant activity showed values comparable to those reported for berries containing anthocyanins (Duymus, Goger, & Baser, 2014; Maatta-Riihinen, Kahkonen, Torronen, & Heinonen, 2005; Zhou et al., 2009). Cyanidin-3-*O*-glucoside, one of the major components of bilberry, is known to reduce ROS formation with radical scavenging activity through inhibition of glutamate-induced Zn^{2+} signalling and through antioxidant and anti-inflammatory mechanisms (Anwar et al., 2014; Cho et al., 2014; Qin, Zhang, & Qin, 2013; Sak, 2014). Delphinidin-3-*O*-glucoside has been shown to prevent hypoxia-induced apoptosis of embryonic cells and acts as a cancer cell invasion suppressor (Im, Jang, Jeong, & Jeong, 2014; Seo et al., 2013). Literature data on the antioxidant activity of spirulina show data comparable to the results obtained in our assays (Nouri & Abbasi, 2018; Takyar, Khajavi, & Safari, 2019). However, our results show that the chemical antioxidant assays provided contrasting results, with a wide variability between ABTS and DPPH assays.

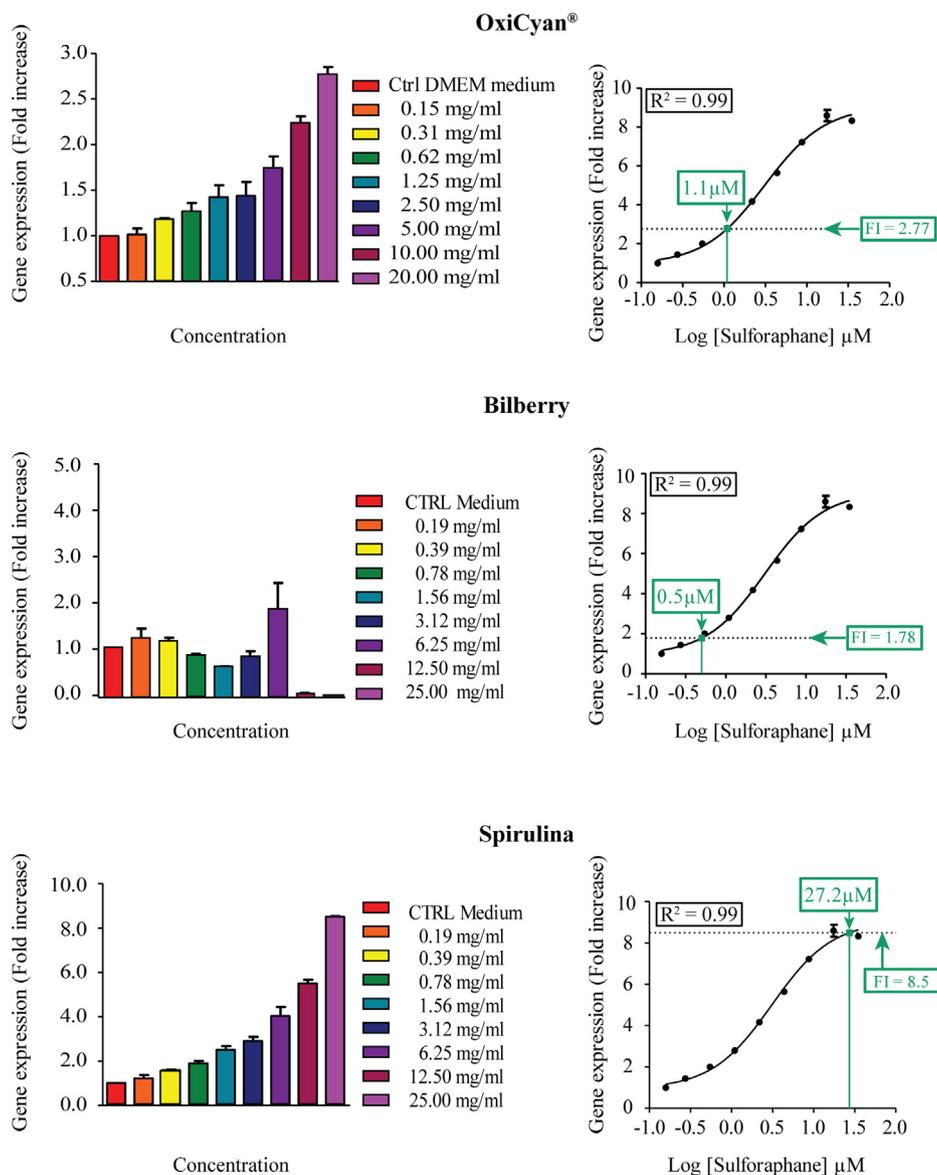Having assessed the chemical antioxidant capacity of OxiCyan®, we

Fig. 2. Effect of OxiCyan®, *Spirulina platensis* (Spirulina) and *Vaccinium myrtillus* (Bilberry) extracts on HepG2 cell natural antioxidant defense. In the left panel, luminescence values reveal luciferase gene expression following ARE promotion. Results are presented as gene expression fold increase with respect to control values. The right panel shows a comparison between the extracts and sulforaphane activity. OxiCyan® exerts an antioxidant promotion equivalent to the EC_{23} (1.1 μM) of sulforaphane, with a gene expression fold increase (FI) 2.77 times with respect to control. Bars indicate standard deviation.

tested its potential radical scavenging activity by using the LUCS technology on HepG2 cells. LUCS assay measures the ability of an antioxidant to neutralize the oxidative stress at the cellular level (Derick et al., 2017). LUCS is based on the production of cellular radical species following the addition in the human hepatocellular carcinoma (HepG2) cells culture medium of thiazole orange, a photo-inducible fluorescent nucleic acid biosensor. The effect of light application at a wavelength of 480 nm in presence of the cellular biosensor triggers the production of singlet oxygen which in turn causes the production of ROS in a biochemical cascade linked to an increase of emitted fluorescence (see also Supplementary Fig. S2). The effect is measured by a delay in the kinetic evolution of fluorescence emission (Derick et al., 2017). The approach has been standardized on high throughput 96-well plates to allow reliable statistical analyses. The ability of OxiCyan® to directly quench HepG2 ROS is shown in Table 5 and Fig. 1. The data partly confirm our chemical assays and indicate a direct antioxidant activity only for OxiCyan® and bilberry, whereas no antioxidant activity was detected at the cellular level for spirulina. Our data are in line with previous studies

showing that HepG2 cells are protected by the antioxidants contained in bilberry extracts, including anthocyanins (Bornsek et al., 2012).

The nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2) is a member of the cap 'n' collar (CNC) subfamily of basic region leucine zipper (bZip) transcription factors (Ma, 2013). Although not essential for blood cell differentiation, Nrf2 mediates induction of a set of drug-metabolizing enzymes by antioxidants and electrophiles (Itoh et al., 1997). For their induction, a DNA sequence known as antioxidant response element (ARE) leads to the detoxification and elimination of several exogenous and endogenous ROS. Nrf2 acts as a xenobiotic-activated receptor to regulate the adaptive response to oxidants and electrophiles (Ma, 2008). Thus, Nrf2 plays a major role in resistance to oxidative stress (Ma, 2013). We then evaluated whether the antioxidant power of OxiCyan® was also related to the ability of its constituents to activate the cell natural defenses. OxiCyan® showed moderate to strong effect on natural antioxidant cell defense with a maximum at 20 mg ml^{-1} (2.77 times the gene expression obtained in control conditions). Induction of ARE/Nrf2 pathway was significant in the range

between 0.31 and 20 mg ml^{-1}. Comparison of OxiCyan® vs sulforaphane (a classical positive control of ARE/Nrf2 pathway (Soane, Li Dai, Fiskum, & Bambrick, 2010)) revealed that OxiCyan® exerted a cell effect equivalent to the EC$_{23}$ (1.1 μM) of sulforaphane (Fig. 2). Although anthocyanins have been found to downregulate polycomb group (PcG) proteins, leading to a subsequent up regulation of caspase-3 by a redox-sensitive mechanism (Leon-Gonzalez et al., 2018), bilberry showed little (or no) effect on natural antioxidant cell defense (ARE/Nrf2 pathway) on the same cell model. The drop of gene expression at 25 mg ml^{-1} can be interpreted as a toxic effect after the 17 h of treatment, which is rather classical for such a long term treatment (Fig. 2). On the other hand, in contrast to its poor chemical antioxidant activity, spirulina showed a strong and dose-dependent effect on ARE/Nrf2 pathway. Comparison of spirulina (25 mg ml^{-1}) vs sulforaphane revealed a cell effect equivalent to the EC$_{97}$ (27.2 μM) of sulforaphane. EC$_{50}$ was not calculated because no maximum effect was reached (no sigmoid fit) (Fig. 2). The effect of spirulina on HepG2 cells has been shown in terms of antiproliferative properties (Abu Zaid et al., 2015; Wu, Ho, Shieh, & Lu, 2005) and chemoprevention of liver toxicity (Ismail et al., 2009). In HepG2 cells, C-Phycocyanin, one of the main phycobiliproteins of spirulina, improved glucose homeostasis in high glucose-induced insulin resistant HepG2 cells (Ren et al., 2018) and modulated a HepG2 multidrug resistance-1 poly glycoprotein (MDR1) by the down regulation of reactive oxygen species and COX-2 pathways via the involvement of NF-κB and AP-1 (Nishanth et al., 2010).

4. Conclusions

The results of this work indicate that OxiCyan®, a phytocomplex composed of bilberry and spirulina, shows a consistent antioxidant power when tested with both chemical and cellular-based assays. OxiCyan® acts on HepG2 cells in a dual mode by both exerting a direct effect on ROS scavenging and as a cytoprotective agent via induction of ARE/Nrf2 pathway. A comparative analysis revealed that the direct ROS scavenging activity of OxiCyan® depends on the anthocyanin moiety provided by bilberry, whereas the gene activation of the ARE/Nrf2 pathway was triggered by the spirulina chemical constituents. The comparative analysis of chemical and cellular-based assays indicated that the antioxidant power evidenced by the chemical assays (e.g., DPPH and ABTS assayed on spirulina) were no longer present in HepG2 cell assays, thus supporting the criticism on these chemical methods when compared to cellular-based methods (EFSA-NDA-Panel, 2017; Schaich, Tian, & Xie, 2015). Taken together, the quantitative results here reported may be used as cell-based reference to improve preparation processes of samples based on OxiCyan® and provide evidence of its antioxidant power at the cellular level.

5. Ethics statement

No humans or animals were used in this work.

Acknowledgements

Declaration of Competing Interest

MEM is also currently a fellow of the Biosfered S.r.l. company. No patents are pending and Biosfered supported the study by providing OxiCyan® and other chemical reagents and partly supporting the research. Biosfered did not interfere with the design, analysis, and decision to publish this paper. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2019.103508.

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