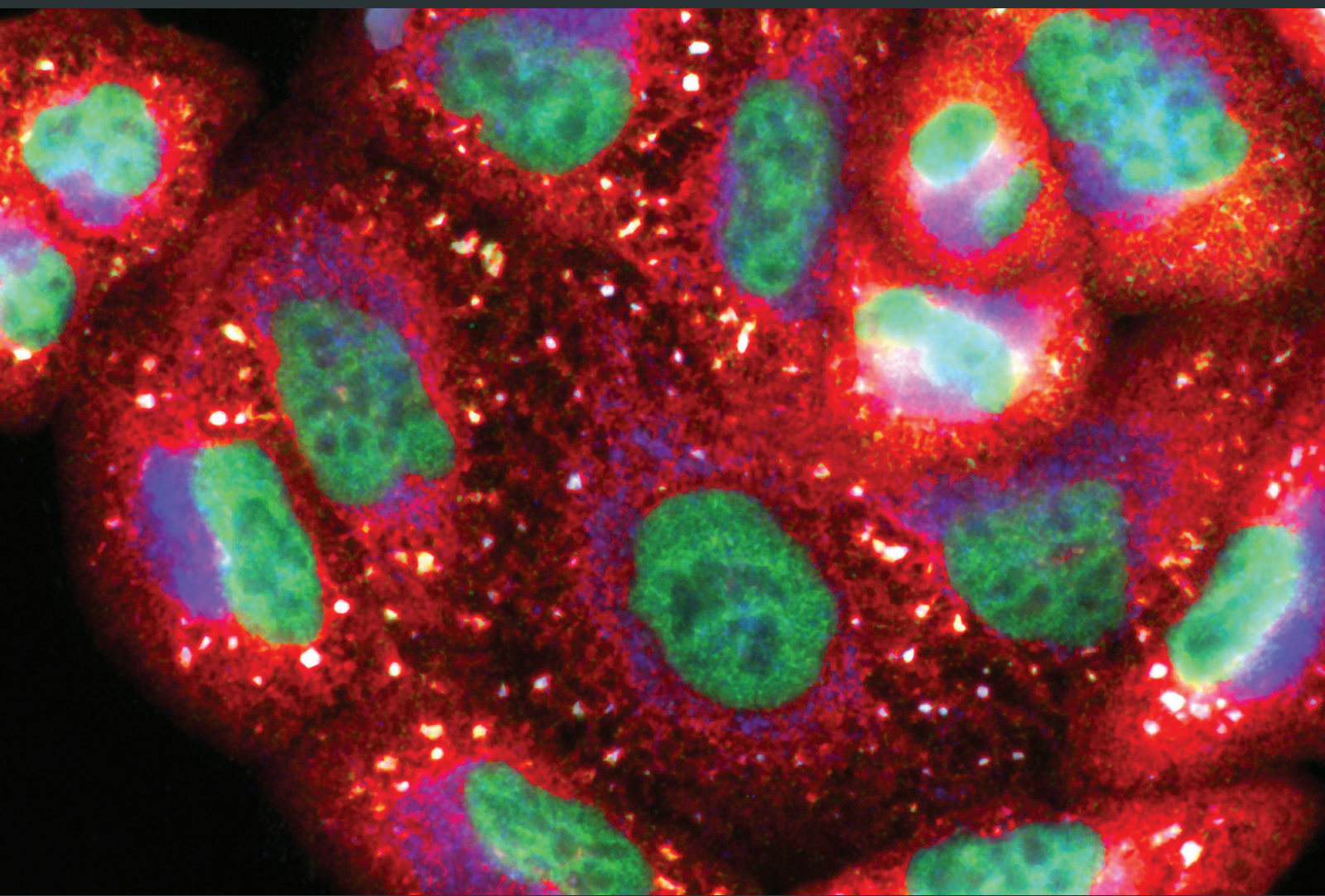
Oxidative Medicine and Cellular Longevity

Biomarkers of Oxidative Stress in Experimental Models and Human Studies with Nutraceuticals: Measurement, Interpretation, and Significance 2017

Lead Guest Editor: Ilaria Peluso
Guest Editors: Maura Palmery and Gregor Drummen



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Editorial Board

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Editorial

Biomarkers of Oxidative Stress in Experimental Models and Human Studies with Nutraceuticals: Measurement, Interpretation, and Significance 2017

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Oxidative stress, the imbalance between reactive oxygen species (ROS) formation and enzymatic and nonenzymatic antioxidants, is involved in the pathogenesis and progression of many ageing-associated diseases, such as cardiovascular disease and certain forms of cancer and neurodegenerative diseases. Many mechanisms and molecular pathways have been implicated in the redox modulation of carcinogenesis and tumor progression. In this context, S. Wang et al. reviewed the involvement of caveolin-1, a constituent protein of caveolae, in cancer promotion and progression, its redox modulation, and its potential role as target of antioxidants. A significant research effort focused on the determination of the antioxidant capacity of natural products and their mode of antioxidant action by using numerous assays and in vitro and animal models. In addition to the antioxidant activity, the modulation of the expression of genes that are regulated by nuclear factor-erythroid 2-related factor 2 (Nrf2), such as antioxidant enzymes, and nuclear factor-kappa B (NF-κB) have been studied extensively; the latter is distinctively involved in immune and inflammatory responses. Many nutraceuticals exert their anti-inflammatory and antioxidant effects through the inhibition of NF-κB and the activation of Nrf2.

K. Anilkumar et al. evaluated the effect of isoorientin isolated from tubers of *Pueraria tuberosa* on lipopolysaccharide- (LPS-) activated RAW 264.7 cells and on mouse paw edema and air pouch models of inflammation. The authors reported that isoorientin inhibited the nuclear translocation of p65 subunit of NF-κB induced by LPS, cylooxygenase-2 (COX-2), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), 5-lipoxygenase (5-LOX), and interleukin-1-β (IL-1-β) and induced glutathione S-transferase and catalase. D. Beghelli et al. reported immunomodulatory activities for the extract of *Boswellia serrata* (BS), a traditional medicinal plant, which has in vitro antioxidant activity due to its significant phenolic content. In addition, different extracts (oleogum and aqueous) were reported to have different effects on regulatory T cells (Treg) and T helper IL-17- (Th17-) producing cells.

Two studies in this issue evaluated the potential antioxidant and anti-inflammatory effects of natural compounds in humans in relation to metabolic profiles. In patients with metabolic syndrome, the consumption of Goji berry for 45 days improved waist circumference, transaminases, lipid profile, lipid peroxidation, glutathione, and catalase, but no effects were observed on inflammatory markers, including C-reactive protein and TNF-α (S. Zanchet et al.). In a pilot study in overweight/obese women, the consumption of an orange juice rich in anthocyanins over a period of 12 weeks improved LDL-cholesterol but had no significant effects on body weight, blood pressure, insulin resistance, antioxidants, and inflammatory status (E. Azzini et al.). Moreover, the

authors suggested that different grades of obesity resulted in different changes in inflammation biomarkers after orange juice consumption.

Peluso and coworkers performed an initial study to evaluate the rejection by the European Food Safety Administration (EFSA) and the Supplement Information Expert Committee (DSI EC) regarding oxidative stress-related bioremedial claims of tea and more specifically green tea extract (GTE). Their investigation based on the peroxidation of leukocytes index ratio (PLIR) and ferric reducing antioxidant potential (FRAP) largely corroborated the EFSA and DSI EC assessment. Albeit that the concentration-dependent prooxidant capacity of most low molecular weight antioxidants has been known for a long time and oxidative stress-related health claims should always be evaluated with care, further investigations on tea and tea-related extracts should be performed over a wide concentration range and in intact organisms in order to include potential potentiating or attenuating cofactors. Finally, the authors once more confirm the essentiality of uric acid in the first line defence against ROS.

I. Marrocco et al. reviewed the biomarkers of oxidative stress in humans and showed that significant methodological bias must be taken into account when interpreting data from the measurement of reactive species in leukocytes and platelets by flow cytometry, from the evaluation of markers based on ROS-induced modifications, from assays regarding the enzymatic players of redox status, and from the measurement of the total antioxidant capacity of human body fluids.

Although it has been suggested that this bias in each method could be overcome by the evaluation of oxidative stress by using more than one criterion, it must be taken into consideration that the clinical significance of any biomarker of oxidative stress in humans must come from a critical analysis of potential bias and that the choice of marker must be considered in the global index, which should be dictated by the subjects' characteristics and by the study aim and design.

We hope that this issue will stimulate significant discussion on the potential confounding of oxidative stress evaluation and on the clinical significance of oxidative stress biomarkers.

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Ilaria Peluso
Maura Palmery
Gregor Drummen

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Review Article

Measurement and Clinical Significance of Biomarkers of Oxidative Stress in Humans

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Oxidative stress is the result of the imbalance between reactive oxygen species (ROS) formation and enzymatic and nonenzymatic antioxidants. Biomarkers of oxidative stress are relevant in the evaluation of the disease status and of the health-enhancing effects of antioxidants. We aim to discuss the major methodological bias of methods used for the evaluation of oxidative stress in humans. There is a lack of consensus concerning the validation, standardization, and reproducibility of methods for the measurement of the following: (1) ROS in leukocytes and platelets by flow cytometry, (2) markers based on ROS-induced modifications of lipids, DNA, and proteins, (3) enzymatic players of redox status, and (4) total antioxidant capacity of human body fluids. It has been suggested that the bias of each method could be overcome by using indexes of oxidative stress that include more than one marker. However, the choice of the markers considered in the global index should be dictated by the aim of the study and its design, as well as by the clinical relevance in the selected subjects. In conclusion, the clinical significance of biomarkers of oxidative stress in humans must come from a critical analysis of the markers that should give an overall index of redox status in particular conditions.

1. Introduction

The redox equilibrium is important in preserving the correct functionality of cellular vital functions [1]. Oxidative stress is defined as the imbalance in the redox characteristics of some cellular environment which can be the result of either biochemical processes leading to the production of reactive species, exposure to damaging agents (i.e., environmental pollutants and radiations), or limited capabilities of endogenous antioxidant systems [2–4]. Reactive oxygen and nitrogen species (ROS/RNS) produced under oxidative stress are known to damage all cellular biomolecules (lipids, sugars, proteins, and polynucleotides) [5, 6]. Thus, several defense systems have been involved within the cells to prevent uncontrolled ROS increase. These systems include nonenzymatic molecules (glutathione, vitamins A, C, and E, and several antioxidants present in foods) as well as enzymatic scavengers of ROS, with superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) being the best-known defense systems [1].

Mitochondria are the predominant source of ROS in all cell types [7]. Superoxide ($O_2^{\bullet-}$) is mainly generated at the level of the mitochondrial electron transport chain and can be converted to hydrogen peroxide (H_2O_2) by SOD or undergo spontaneous dismutation [1]. In the presence of transition metal ions, for example, iron and copper ions, H_2O_2 can generate via Fenton reaction the highly reactive hydroxyl radical ($HO^{\bullet}$). Reactive species may also be enzymatically produced by xanthine oxidase (XO), uncoupled nitric oxide synthases (NOS), and NADPH oxidase (NOX). ROS production is related not only to cell damage or death, but physiological and signalling roles for ROS have also been ascertained. Reactive species are the principal source of defensive pro-oxidants generated in the respiratory burst of neutrophils [8, 9]. Upon activation, neutrophils produce various ROS via myeloperoxidase (MPO) and RNS via inducible

Table 1: Fluorescent probes used for the measurements of reactive oxygen and nitrogen species by flow cytometry.

Probe (localization)	ROS/RNS	Fluorescence	Leukocytes	Platelets	Limitations and confoundings
DCFH-DA (intracellular)	$HO^{\bullet}$ $ONOO^{-}$ $ROO^{\bullet}$ $NO_2^{\bullet}$ Indirect H_2O_2	↑ green (DCF)	Yes	Yes	Hemolysis Self-propagation of DCF radicals MDR substrates or inducers Esterase inhibitors Plasma esterase in whole blood or PRP EDTA and citrate Antioxidants
DAF-2 DA/DAF-FM DA (intracellular)	$NO^{\bullet}$	↑ green (DAF-Ts)	Yes	No	MDR substrates or inducers Esterase inhibitors Plasma esterase in whole blood
DHR123 (intracellular)	HClO H_2O_2 $ONOO^{-}$	↑ green (Rho123)	Yes	No	Self-propagation of DHR radicals MDR substrates or inducers Antioxidants
HE (intracellular)	$O_2^{\bullet-}$	↑ red (ethidium)	Yes	No	Intercalating agents
C11-BODIPY$^{581/591}$ (membrane)	$HO^{\bullet}$ $ROO^{\bullet}$	Shift from red to green	Yes	Yes	Hemolysis Antioxidants

C11-BODIPY$^{581/591}$: 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid; DAF-2 DA: 4,5-diaminofluorescein diacetate; DAF-FM DA: 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate; DAF-Ts: triazolofluoresceins; DCF: 2′,7′-dichlorofluorescein; DCFH-DA: dihydrochlorofluorescein diacetate; DHR123: dihydrorhodamine 123; EDTA: ethylenediaminetetraacetic acid, H_2O_2: hydrogen peroxide; HClO: hypochlorous acid; HE: hydroethidine; MDR: multidrug resistance; $NO^{\bullet}$: nitrogen monoxide; $NO_2^{\bullet}$: nitrogen dioxide; $O_2^{\bullet-}$: superoxide radical; $HO^{\bullet}$: hydroxyl radical; $ONOO^{-}$: peroxynitrite; PRP: platelet-rich plasma; Rho123: rhodamine 123; $ROO^{\bullet}$: peroxyl radicals.

nitric oxide synthase (iNOS). MPO catalyzes the H_2O_2-dependent formation of hypochlorous acid (HClO) while iNOS produces nitric oxide ($NO^{\bullet}$), which then reacts with $O_2^{\bullet-}$ to form peroxynitrite ($ONOO^{-}$) [10]. NOX associated with cell membrane catalyzes the generation of superoxide radicals that play a physiological role in cancer invasion, hypoxia, and integrin signaling [11–13]. Furthermore, ROS can modulate the expression of several genes through the redox regulation of the nuclear factor-erythroid 2-related factor 2 (Nfr2) and the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) [1, 14]. A concerted modulation of these pathways has been suggested in inflammation and carcinogenesis [14].

During the past decade, research has revealed a widespread involvement of oxidative stress in a number of disease processes, including cancer, cardiovascular disease (CVD), atherosclerosis, diabetes, arthritis, neurodegenerative disorders, and pulmonary, renal, and hepatic diseases [1, 5, 15–23]. These pathologic states have increased incidence with age, and oxidative stress is believed to be a major factor in ageing and ageing-associated diseases [24–26]. Thus, oxidative stress markers are important tools to assess the biological redox status, disease state and progression, and the health-enhancing effects of antioxidants in humans. Identifying markers of oxidative stress has been the focus of many studies, and several markers from various biomolecule sources have been proposed over the past decades. However, for some of them, there is a lack of consensus concerning validation, standardization, and reproducibility. We aim to discuss the major bias of these methods.

2. Measurement of Reactive Species in Leukocytes and Platelets by Flow Cytometry

In humans, under physiological conditions, ROS and RNS generated by leukocytes, through NOX and iNOS, have a role in the innate immune response to infection [8, 9]. However, ROS and RNS can induce lipid peroxidation of polyunsaturated fatty acids (PUFAs), which propagate via peroxyl radicals ($ROO^{\bullet}$) within the membrane, as well as in the low-density lipoproteins (LDL) [5, 2721]. In particular, in the context of metabolic syndrome and chronic inflammation, the oxidized LDL (oxLDL) activate leukocytes and/or platelets to produce ROS and RNS [27–29].

The direct quantification of ROS/RNS is a valuable and promising biomarker that can reflect the disease process. However, given the short half-life of these species, their measurement in biological systems is a complex task. Approaches include electron spin resonance, fluorescence magnetic resonance, and mass spectrometry techniques [30, 31], but their use has been limited to cell cultures and other in vitro applications. Although free radicals' production can be measured by spectrophotometric or luminescence methods [32, 33], all extracellular free radicals' measurements are deeply affected by cell count and viability.

On the other hand, flow cytometry is one of the most powerful tools for single-cell analysis of the immune system [34] and it is routinely used in the diagnosis and progression evaluation of blood cancers [35–38] and human immunodeficiency virus (HIV) infection [39–41]. In addition to the role of oxidative burst evaluation by flow cytometry in the diagnosis of chronic granulomatous disease [42], this instrumentation has been used for many years to evaluate oxidative burst in many conditions, such as autoimmune neutropenia [43] and asymptomatic HIV+ individuals [44].

Many fluorescent probes for the detection of reactive species have been developed in the last years, with a different degree of specificity and sensitivity [45]. The fluorescent probes used for the detection of reactive species in blood cells via flow cytometry are summarized in Table 1.

For instance, intracellularly converted diacetate derivatives of probes such as dihydrochlorofluorescein diacetate (DCFH-DA), 4,5-diaminofluorescein diacetate (DAF-2 DA), and 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA) have widely been used for ROS/RNS detection [32, 33, 45–47]. Once taken up by cells, these probes are hydrolyzed by intracellular esterases, generating the nonfluorescent and membrane-impermeable DCFH, DAF-2, or DAF-FM. Subsequent oxidation by ROS/RNS results in the formation of the fluorescent 2′,7′-dichlorofluorescein (DCF) and triazolofluoresceins (DAF-Ts), respectively.

DCFH, the more commonly used probe, does not directly react with H_2O_2 to form the fluorescent product. DCFH can be instead oxidized to DCF by several one-electron-oxidizing species including $HO^\bullet$ radicals, products formed from peroxidase or heme proteins reacting with H_2O_2, HClO, and nitrogen dioxide ($NO_2^\bullet$) generated by myeloperoxidase and peroxynitrite decomposition. DCFH oxidation can also be promoted by Fe^{2+} in the presence of O_2 or H_2O_2. The 1-electron oxidation of DCFH generates the DCF semiquinone anion radical ($DCF^{\bullet-}$). This intermediate can rapidly react with O_2 to form $O_2^{\bullet-}$, which in turn can dismutate yielding additional H_2O_2 and establishing a redox-cycling mechanism that leads to an artificial amplification of the fluorescence signal [46].

While DCFH is used in both platelets and leukocytes, dihydrorhodamine 123 (DHR123) and hydroethidine (HE) are used only in the evaluation of the oxidative burst by polymorphonuclear leukocytes (PMN) (Table 1).

DHR123 is an uncharged nonfluorescent probe that passively diffuses across cell membranes and is converted upon oxidation to the fluorescent membrane-impermeant rhodamine 123 (Rho123), which predominantly localizes in the mitochondria [32, 33, 45, 47]. HE passively diffuses into cells and is preferentially oxidized by $O_2^{\bullet-}$ to ethidium, which results in intercalation in DNA and consequently a significant enhancement of its red fluorescence intensity [32, 33, 45, 47].

The 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (C11-BODIPY$^{581/591}$) probe is the only lipophilic probe used to evaluate ROS in leukocytes and platelets [48, 49]. C11-BODIPY$^{581/591}$ is a derivatized 11-carbon fatty acid in which the boron dipyrromethene difluoride (BODIPY) core is substituted by a phenyl group via a conjugated diene [50, 51]. This conjugated diene interconnection is oxidation sensitive, and when oxidized by $HO^\bullet$ or $ROO^\bullet$, disruption and shortening of the conjugated electron resonance structures between the phenyl group and the BODIPY core shifts C11-BODIPY$^{581/591}$'s fluorescence from red to green [50, 51]. Conversely, $ONOO^-$ induces not only oxidation but also nitration of BODIPY, reducing red fluorescence but not necessarily increasing green fluorescence [52]. Although excimers of the oxidized form are red fluorescent, labelling conditions up to 30 μM provides sufficient staining of the plasma and organelle membranes well below the range in which self-quenching or excimer formation occurs [51]. Therefore, excimers do not interfere with the fluorescence of BODIPY and the measured red signal depends only on the reduced form of the probe. Furthermore, neither C11-BODIPY$^{581/591}$ nor its oxidation products are able to spontaneously leak from the lipid bilayer [51] and the ratio of oxidized to nonoxidized C11-BODIPY$^{581/591}$ can be used to normalize probe incorporation in cells of different size (lymphocytes, monocytes, and granulocytes) [49]. Only hemolysis and antioxidants, in particular the end-product of purine metabolism, uric acid (UA), could bias the measurement of ROS generation [49, 53].

On the contrary, when analyzing the results of intracellular probes, many factors must be taken into account (Table 1).

Ethidium displacement by molecules, such as chemotherapeutics [54] or flavonoids [47], could decrease the ethidium fluorescence signal, making the interpretation of data difficult.

Artefactual amplification of the fluorescence intensity has been suggested to occur via intermediate radicals for both DCF and DHR [46], whereas the presence of quenching and reducing antioxidants could either decrease [55] or increase [56] the oxidation of probes without affecting ROS production. Heme proteins and reduced iron (Fe^{2+}) have been shown to oxidize DCFH, and the suitability of DCFH-DA for measuring intracellular ROS is increasingly being questioned [46].

In addition to the aforementioned limitations, the fluorescence signal is dependent not only on the oxidation of the probe but also on its concentration. In this context, multidrug resistance- (MDR-) mediated transport has low substrate specificity. Multidrug resistance-related protein 2- (MRP2-) mediated DCF extrusion has been reported in human leukocytes [57], and it is well known that Rho123 can be extruded by the MDR [47]. The inclusion of H2DCF-DA in the dilution buffer in order to avoid dye leakage has been suggested [58]. However, overloading with probe generates cell morphology changes and artifacts in platelets [59, 60]. In this context, it should be pointed out that lyophilic derivatives of intracellular fluorescent probes are substrates of P-glycoprotein (Pgp) and MRP1 [47]. Furthermore, MDR expression is affected by intracellular variation of glutathione (GSH) [61] and oxidative stress [62–65], as well as by various dietary factors [66–69], inflammatory cytokines [70–72], disease states [73–75], and drugs [76–81]. In particular, aspirin, indomethacin, and ibuprofen are substrates for MRP4 [76] and may interfere with fluorescent probe staining. Most importantly, aspirin treatment over a period of 15 days significantly increased MRP4 mRNA and protein expression in platelets of healthy volunteers [78]. MRP4 is involved in the storage of cyclic nucleotides in dense granules [82–84], and MRP4 inhibition impairs platelet aggregation [85]. Besides the aforementioned effects, MRP4 also has a role, together with MRP1 [86], in the efflux of leukotrienes [87]. Therefore, in addition to the potential confounding effect on the fluorescence signal [88], the presence of intracellular probes per se could reduce platelet activation in vitro.

In addition, intracellular esterase activity was shown to be impaired in damaged platelets and highly correlated with ADP-induced aggregation [89], whereas plasma esterases [59, 60] and/or inhibition of esterases [47] could potentially interfere with probe staining and fluorescence signal

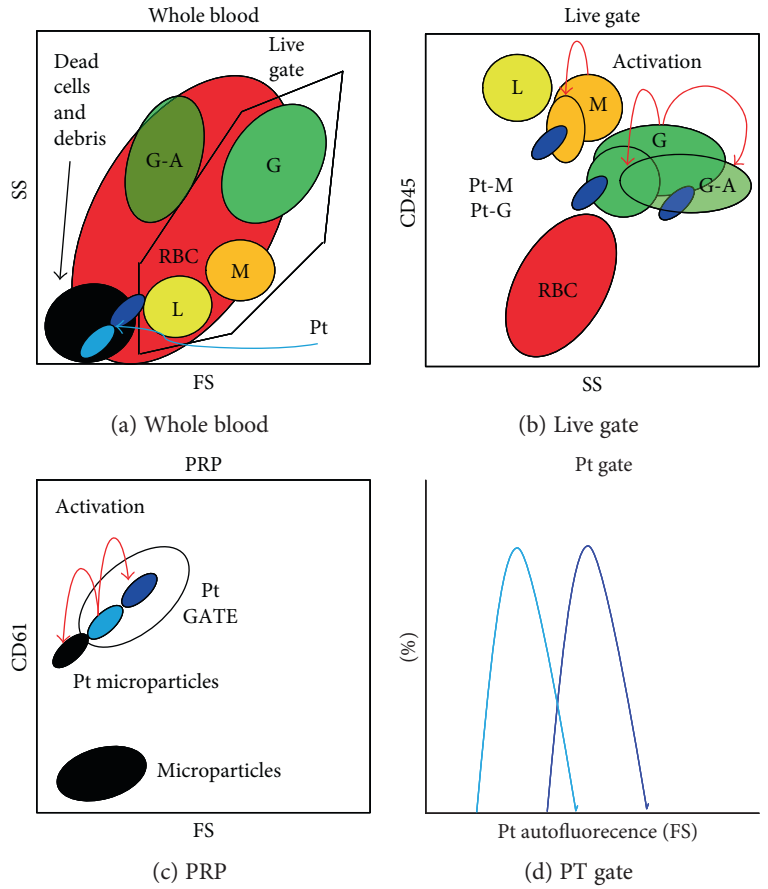

Figure 1: Gating strategies in the measure of free-radical production by flow cytometry. Different leukocytes populations (lymphocytes: L, monocytes: M, and granulocytes: G) in whole blood can be identified by CD45 (b) in the live gate assigned in the forward scatter (FS) and side scatter (SS) dot plot (a) by excluding dead cells and debris. Red blood cells (RBC) can be excluded as CD45 negative (b). Platelets (Pt) can be identified by CD61 in platelet-rich plasma (PRP) (c). In activated samples, platelet microparticles (c) and leukocyte-platelet aggregates (b: Pt-G and Pt-M) are formed and Pt-G are more prone to apoptosis (G-A). After platelet activation, FS increases due to platelet aggregation inducing an increase in autofluorescence (d).

intensity when using DCFH-DA, DAF-2 DA, and DAF-FM DA in whole blood and platelet-rich plasma (PRP) methods.

Despite the fact that whole blood methods provide more physiologically relevant data when evaluating ROS production in leukocytes [32], washing impairs ADP-induced aggregability of platelets [90] and alters their structure [91], whereas ethylenediaminetetraacetic acid (EDTA) and citrate increase DCFH oxidation [56].

Moreover, whole blood, PRP, and platelet-poor plasma [92] also contain XO, and therefore, UA may be produced during the incubation period with ROS-inducers, potentially falsifying results. Urate crystals induced oxidative burst [27] and the activation and lysis of platelets in vitro [93, 94].

With this in mind, it is well known that there is an increased platelet destruction and production in some patients with primary gout [95, 96] and that platelet apoptosis and microparticles derived from platelets, erythrocytes, leukocytes, and/or endothelial cells are higher in subjects with CVD [97–99], dyslipidemia [100], and metabolic syndrome [101]. On the other hand, lipid-lowering treatment [100] and the XO inhibitor febuxostat [102] were shown to decrease microparticle count. Gender differences have been reported for microparticle count. Specifically, higher levels of microparticles have been found in women compared with men [103]. Endotoxin induced the formation of platelet microparticles [104], introducing potential confounding factors in conditions of increased levels of lipopolysaccharide, such as the postprandial state [105] and metabolic [106] and inflammatory diseases [107]. Spontaneous activation, generating both microparticles and inducing microaggregation of platelets, occurs in type 2 diabetic patients [108], increases with age in healthy subjects [109], and is affected by blood collection and processing procedures [109, 110]. On the other hand, platelet aggregates with leukocytes are a marker of activated platelets in CVD patients [111–114], potentially reducing the platelet count in PRP. All these factors must be taken into account when evaluating data from case-control studies that compared ROS-production in unstimulated samples of disease and healthy subjects.

The combination of fluorescently labeled antibodies against targets such as the pan-leukocyte marker CD45 [49] and the platelet marker CD61 [48] and/or physical properties such as size (FS: forward scatter) and internal complexity (SS: side scatter) can identify different leukocyte populations and platelets (Figure 1).

Table 2: Markers based on ROS-induced modifications.

Markers	Methods	Limitations and confoundings
Lipid oxidation		
HNE	HPLC, GC-MS Immunoassay	
MDA, alkenals, alkadienals	Spectrophotometric/fluorimetric (TBARS), HPLC (UV or fluorescence) Immunoassay	Sugars, amino acids, bilirubin and albumin, hemolysis
F2-IsoPs	Gas/liquid chromatography coupled with mass spectroscopy techniques Immunoassay	Hemolysis Antibody specificity
DNA oxidation		
8oxodG, 5-chlorocytosine, 5-chlorouracil, εdA, εdC	ELISA assays, HPLC-ECD, HPLC/GC-MS	Antibody specificity
Protein oxidation		
ALEs, AGEs	HPLC, Western blot after one-dimensional or two-dimensional electrophoretic separation, immunohistochemistry, ELISA	Structural heterogeneity of these products Antibody specificity
Carbonils	Spectrophotometric, HPLC, ELISA	
3-NO-Tyr	HPLC/GC-MS, ELISA	Possible nitration of tyrosine residues in the sample by the presence of nitrite and the acid conditions during protein precipitation and hydrolysis Antibody specificity
AOPP	MS, colorimetric assays	
oxLDL	Immunodetection (ELISA)	Antibody specificity
IMA	ABC test, immunodetection (ELISA)	Sensitive to pH changes, temperature, and time of sample storage Antibody specificity

8oxodG: 7,8-dihydroxy-8-oxo-2′-deoxyguanosine; ABC test: binding capacity of albumin for cobalt; AGEs: advanced glycation end products; ALEs: advanced lipoxigenation end products; AOPP: advanced oxidation protein products; F2-IsoPs: F2-isoprostanes; GC: gas chromatography; HNE: 4-hydroxy-2-nonenal; HPLC: high-performance liquid chromatography; ECD: electrochemical detection; IMA: ischemia-modified albumin; MS: mass spectroscopy; MDA: malondialdehyde; TBARS: thiobarbituric acid reactive substances.

In activated samples, platelet microparticles [103, 104, 115], platelet aggregates [116], and leukocyte-platelet aggregates [117, 118] are formed (Figure 1). In particular, platelet activation in whole blood induces the formation of platelet conjugates with granulocytes or monocytes [119] and leukocyte aggregates with platelets are more prone to apoptosis after in vitro activation (Figure 1) [117].

Regarding the normalization strategies, stimulation indexes calculated from the mean intensity fluorescence (MIF) values and expressed as fold change relative to unstimulated samples have been suggested for evaluating the production of ROS in both granulocytes [120, 121] and platelets [122, 123]. However, these methods do not take into account probe leakage nor autofluorescence differences. While it is well known that autofluorescence generates false-positive monocytes [124], this aspect is neglected in platelet assays. Despite controversy regarding the relationship between CVD and platelet size, measured as mean platelet volume (MPV) or FS [97, 125, 126], it is well known that FS increases after platelet activation [127] and that large and small platelet subpopulations have different autofluorescence profiles [128] (Figure 1). Consequently, differences in autofluorescence in unstimulated and stimulated samples imply that stimulation indexes do not necessarily measure ROS production. Therefore, it must always be taken into account that the fluorescence signals and not the radicals are measured and that the oxidation of the probe is not always related to ROS production. Overall, the reviewed potential bias and confounding factors suggest that accurate gating and normalization strategies must be applied in order to avoid misinterpretation of the results.

3. Markers Based on ROS-Induced Modifications

In addition to the measure of free-radical production, a different approach is measuring stable markers that may reflect a systemic or tissue-specific oxidative stress. Such molecules are modified by the interaction with ROS in the microenvironment [129] (Table 2).

Lipids, DNA, and proteins are examples of molecules that can be modified by excessive ROS in vivo (Table 2) [129]. Some of these modifications are known to have a direct effect on the function of target molecules, such as the inhibition of an enzymatic function, but other modifications just reflect the local degree of oxidative stress. This influences the clinical applicability of several oxidative stress markers since the functional significance or the causal role of oxidative modifications on biological functions is a key characteristic for the validity of a biomarker (Table 2).

While measures of oxidative stress in spinal cord [130] and tissues [131, 132] are restricted to particular disease conditions, venous blood and urinary samples are the most commonly used in clinical practice. In addition to urinary samples [133–135], other noninvasive and low-cost tools for the screening of oxidative stress, such as salivary

[136–138] or exhaled breath [139–141] analysis, have been proposed. However, it has been reported that creatinine urinary markers are not suitable in patients with impaired renal function [135]. Therefore, the validity of a biomarker depends on the choice of the sample that should be dictated by subjects' characteristics and the best cost-benefit ratio.

3.1. Lipid Oxidation Products. Lipid oxidation end product determination is a widely used marker of oxidative stress.

The presence of unsaturated double bonds makes PUFAs, mainly arachidonic acid (AA), highly susceptible to oxidative damage in the presence of ROS or free radicals [5]. Lipids peroxidation may also occur through enzymatic reactions, catalyzed by lipooxygenase and cyclooxygenase (COX), which oxidize AA into prostaglandins, prostacyclin, tromboxane, and leukotrienes. Free radical-mediated oxidation involves an autocatalytic chain reaction triggered by ROS, mainly $HO^{\bullet}$ and $ROO^{\bullet}$, which catalyze a hydrogen-atom subtraction at the unsaturated bonds generating a carbon radical that can further react with oxygen producing a lipid peroxyl radical. The chain reaction proceeds with lipid peroxyl radical acting as chain-carrying radicals and the formation of lipid hydroperoxydes. In the presence of transition metals, lipid hydroperoxides may generate lipid alkoxyl and $ROO^{\bullet}$ as well as $HO^{\bullet}$, which can further sustain the chain oxidation reaction to produce short-chain oxidation products, including a variety of different aldehydes, alkanes, and alkenes. Malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE) represent the most investigated end product of lipid oxidation [142]. HNE can be detected by high-performance liquid chromatography (HPLC) directly or as a derivatized product with 2,4-dinitrophenylhydrazine or 1,3-cyclohexanedione, by gas chromatography coupled with mass spectroscopy (GC-MS), and by means of immunological techniques using specific anti-HNE antibodies [142–144]. However, when 4-HNE aldehydes were determined using GC-MS system, they were significantly different in plasma and urine of patients with rheumatoid arthritis compared to healthy subjects, but differences between patients with low and high disease activity can be detected only in plasma samples, suggesting that only this sample is useful to monitor the progression of this autoimmune disease [145].

MDA, alkenals, and alkadienals constitute the thiobarbituric acid reactive substances (TBARS), which can react with two equivalents of thiobarbituric acid (TBA) to give a pink adduct complex, easily measured by a colorimetric or fluorimetric assay (Table 2). Despite TBA test for MDA determination being the most frequently used method to evaluate lipid peroxidation, it shows several pitfalls and has been criticized as being too unspecific and prone to artifacts [146–148]. TBA can react with several compounds, including sugars, amino acids, bilirubin, and albumin, producing interferences in the measurement (Table 2). There is a further MDA generation, which occurs during the procedure itself that may be prevented by adding an antioxidant, like butyl hydroxytoluene (BHT), and by reducing the heating time. An additional pitfall is the interference of hemolysis that falsely increases the measured MDA levels (Table 2). Thus, many protocols and modifications of the TBA test are available in the literature, and while direct MDA-TBA adduct measurement has a low significance, the determination by HPLC combined with UV or fluorescence detection is a more reliable and reproducible method [149–151]. Despite the methodological bias, MDA measurement could have clinical relevance due to the potential pathogenic role of MDA on to the induction of IL-17 producing cells [152] and a possible link between lipid-peroxidation and T-helper 17 (Th17) cell-mediated diseases, such as inflammatory bowel diseases [153].

F2-isoprostanes (F2-IsoPs), chemically stable prostaglandin-like isomers generated by the reaction of polyunsaturated fatty acids in membrane phospholipids and free radicals or ROS, represent another reliable marker assessing oxidative stress status in vivo [154–156]. In fact, they are initially formed in lipid membranes as a consequence of oxidative stress and then released in free form by phospholipase action. F2-IsoPs are unaffected by lipid content in diet and thus their measurement in biological fluids as well as exhaled breath condensate can provide an estimation of total body production, whereas measurement of F2-IsoPs esterified in tissues of interest can provide information to localize and quantify the specific oxidative stress. Despite these observations, the utility of F2-IsoPs as biomarkers of oxidative stress is highly limited since their reliable quantification is costly requiring gas/liquid chromatography coupled with mass spectroscopy techniques (HPLC/GC-MS). It must be taken into account that also measures of both MDA and 15(S)-8-iso-PGF(2alpha) by GC-MS/MS in plasma samples may be markedly compromised by hemolysis [154]. Immunoassay techniques, based on specific antibodies, are under development, but their application is limited since the obtained results do not correlate well with mass spectrometry determination [155–157]. In addition to the methodological considerations, it must be taken into account that in some inflammatory conditions, the enzymatic product of arachidonic acid prostaglandin F2α (PGF2α) must be evaluated with nonenzymatic oxidation products (F2-IsoPs) in different tissues [158]. In fact, it has been recently reported that PGF2α levels, but not F2-IsoPs, were higher in cerebrospinal fluid of patients with multiple sclerosis (compared with controls); however, in plasma, both F2-IsoPs and PGF2α were lower in patients with progressive disease and decreased with increasing disability score [158]. A good approach could be to study the profiling of eicosanoid metabolome, as recently suggested in animal models of rheumatoid arthritis [159, 160].

3.2. Markers of DNA Oxidation. Oxidation of DNA components by ROS/RNS is the major source of induced DNA damages leading to several types of DNA modifications including nucleotide oxidation, strand breakage, loss of bases, and adduct formation [161, 162]. The $HO^{\bullet}$ radical can react with all purine and pyrimidine bases, as well as deoxyribose backbone, generating various products, the most common one being 7,8-dihydroxy-8-oxo-2′-deoxyguanosine (8oxodG) [163].

Oxidatively generated lesions can lead to decomposition in base fragments and the formation of carbon-centered radicals, which give rise, in most cases, to DNA strand breaks. Exposure of DNA to RNS can promote deamination

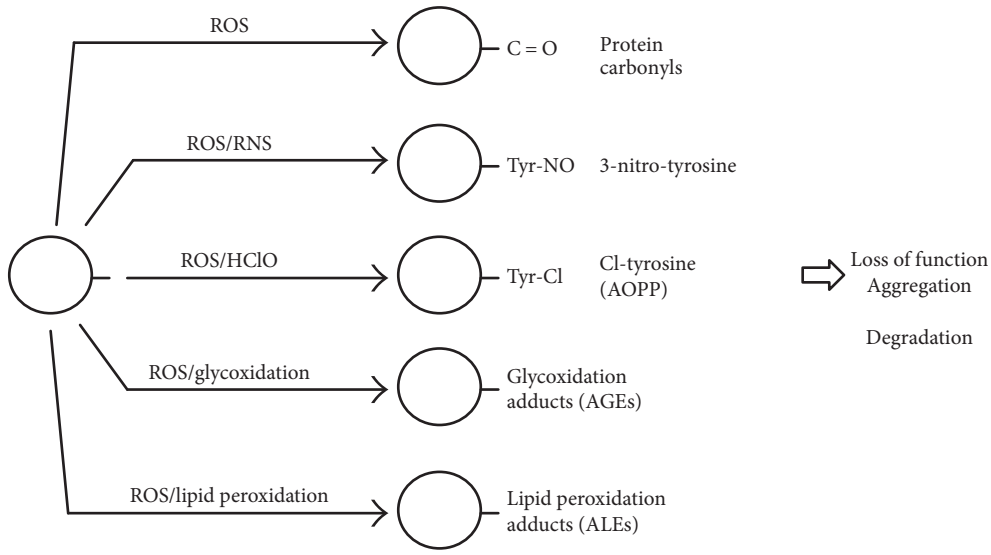

Figure 2: Irreversible oxidative modifications of proteins. AGEs: advanced glycation end products; ALEs: advanced peroxidation end products; AOPP: advanced oxidation protein products; HClO: hypochlorous acid; RNS: reactive nitrogen species; ROS reactive oxygen species.

of DNA bases and conversion of guanine into xanthine, oxanine, and 8-nitroguanine, which is rapidly lost from DNA by spontaneous depurination. The major end products of HClO include 5-chlorocytosine and 5-chlorouracil. These modified bases have been detected at sites of inflammation and are indicative of HClO-mediated DNA damage in vivo [164].

DNA damage may also be caused by the attack of reactive products resulting from ROS-induced modifications of other molecules, such as lipids. In this case, etheno-DNA adducts, such as 1,N(6)-etheno-2′-deoxyadenosine (εdA) and 3,N(4)-etheno-2′-deoxycytidine (εdC), are formed and can be used as biomarkers of oxidative stress [165] and may serve as potential markers for assessing progression of inflammatory cancer-prone diseases [166]. Elevated etheno-DNA adducts were found in tissues of patients suffering from chronic inflammatory processes [167] while increased levels of urinary εdA were observed in subjects and workers exposed to diesel engine exhaust [168]. Etheno-DNA adducts can be measured by HPLC/MS-based techniques [165, 169].

It has been estimated that several thousands of 8-oxodG lesions may form daily in a mammalian cell, representing 5% of all oxidative lesions, and for this reason, 8-oxoG is the most commonly used biomarker of DNA oxidation to measure oxidative stress [170–172]. However, analysis of 8-oxodG and other oxidized purines and pyrimidines has been hampered for a long time by the occurrence of several drawbacks associated with their measurement. Optimized assays are now available, and the most reliable is represented by chromatography coupled with mass spectroscopy, even if commercial ELISA assays based on specific antibodies are available [173, 174]. Although ELISA methods are less specific compared to HPLC with electrochemical detection (HPLC-ECD) and HPLC/GC-MS, some kits with specific antibodies resulted appropriate for urine samples [175].

The oxidized nucleotides are excreted into the urine, and their measurement has been proved to be predictive of the development of several diseases. High level of DNA oxidation, measured as urinary excretion of 8oxodG, is predictive for the risk of breast and lung cancer, atherosclerosis, and diabetes [176–179]. RNA oxidation, measured as 7,8-dihydroxy-8-oxoguanosine (8oxoGuo), has been recently introduced as a marker of diseases, particularly neurodegeneration and diabetes, and high level of RNA oxidation has been also associated with breast cancer development in diabetic females [180].

3.3. Protein Oxidation Products. Proteins represent a wide target for ROS and RNS generated under normal or oxidative stress conditions and can be considered as general scavengers of these species. Several amino acidic residues can undergo oxidative modifications including oxidation of sulphur-containing residues, hydroxylation of aromatic and aliphatic groups, nitration of tyrosine residues, nitrosylation and glutathionylation of cysteine residues, chlorination of aromatic groups and primary amino groups, and conversion of some amino acid residues to carbonyl derivatives [181, 182] (Figure 2).

Oxidation can also lead to the cleavage of the polypeptide chain and to the formation of cross-linked protein aggregates [183, 184] (Figure 2).

Oxidation of iron-sulphur centers by $O_2^{\bullet-}$ is irreversible and leads to enzyme inactivation. In addition, metals bound to the protein can generate, through the Fenton reaction, $HO^{\bullet}$ radicals that rapidly oxidize the neighbor amino acid residues of the protein [185].

If the oxidative modifications of protein residues are not properly repaired or removed, they could affect the three-dimensional structure and physicochemical properties of the protein that may also become toxic.

Irreversible modifications of proteins include carbonylation, nitrosilation, breaking of the histidine and tryptophan rings, and hydrolysis of the peptide bond in the presence of proline [186]. The latter mainly occurs in the collagen, rich in proline and hydroxyproline, which is particularly damaged under oxidative stress conditions [187].

Determination of protein oxidation has a biological significance and a good clinical relevance. A specific profile of oxidized proteins may be formed as a consequence of different oxidative stress or age-related diseases [188–190]. Biological significance of protein oxidation may also result from its chemical stability and high yield formation. Sample availability is an important factor that limits the reliability of a biomarker. Protein oxidation may be determined in blood and urine samples, although determination in specific tissue or cell samples may give more precise information. It must be noted that protein oxidation may occur during the analytical process thus generating some artefacts [191]. The rates of oxidation reactions are critically dependent on the sample temperature, its physical form, and the presence of oxygen and catalysts (metal ions and light) [192]. For these reasons, measurement of protein oxidation may be a useful marker, as long as it is characterized by a high reproducibility, sensitivity, and specificity.

Several methods have been developed for the detection of the different kinds of protein modifications. However, the biological and clinical relevance of protein oxidation as a biomarker is still limited by the availability of methodologies able to identify and quantify specific protein oxidative modifications.

3.3.1. Protein Carbonyls, ALEs, and AGEs. Carbonyl groups can be generated by many different mechanisms, as the oxidative cleavage of the protein backbone, in particular at the level of glutamyl side chains, and the oxidative deamination of lysine. Also, the attack of $HO^{\bullet}$ radicals on proline, lysine, arginine, and threonine side chains generates carbonyl groups [193].

The measure of carbonyl levels in proteins is the most widely used marker of oxidative protein damage, and tissues injured by oxidative stress generally contain increased concentrations of carbonylated proteins [186, 194]. Moreover, this biomarker has some advantages in comparison with the measurement of other oxidation products because of the relative early formation and the relative stability of carbonylated proteins. Protein carbonyl levels increase with age and are elevated in several pathologic conditions including neurodegenerative diseases, obesity, or diabetes [195, 196].

Methods based on ELISA and HPLC are the most used in clinical assessments because of high throughput and standardization. Detection of protein carbonyl groups generally involves the derivatization of the CO group with 2,4-dinitrophenylhydrazine (DNPH) with the formation of a stable dinitrophenyl (DNP) hydrazone product. The latter can be detected by several methods which include the direct spectrophotometric measurement of DNP adducts, as well as more specific techniques based on anti-DNP antibodies, like ELISA, Western blot after one-dimensional or two-dimensional electrophoretic separation, immunohistochemistry, and HPLC [197–199].

Functional groups of proteins can react with several products resulting from the ROS-induced oxidation of PUFAs and carbohydrate, generating inactive adduct derivatives classified as advanced peroxidation end products (ALEs) and advanced glycation end products (AGEs), respectively [200, 201] (Figure 2). In particular, lysine, histidine, and cysteine residues can react with lipid peroxidation products (HNE, MDA), through a Michael addition reaction, while lysine ε-amino groups can react with reducing sugars and their oxidative products, to generate several carbonyl derivates [202, 203].

AGEs are a heterogeneous group of molecules with carboxymethyl lysine, carboxymethyl valine, and pentosidine as the main protein products, while carboxymethyl lysine is a product of both lipid peroxidation and glycoxidation reactions [204–206].

AGEs increase with aging and their formation has been related to the level of carbohydrates; so, they have been linked to diabetes and obesity [207], as well as other diseases including atherosclerosis, Alzheimer's disease, and renal insufficiency [208, 209]. Mass spectrometry-based techniques represent a key method in identifying protein adducts and the specific site of modification but their use is still limited in routine clinical analysis [210, 211]. To address this, AGEs' assays are mostly based on the use of specific antibodies or spectrofluorimetric measurements based on the fluorescent properties of AGEs [212, 213]. Although promising results came from studies on skin autofluorescence in diabetic patients [214, 215], the serum fluorescence AGE (F-AGE) method did not distinguish women with gestational diabetes from the healthy controls [216].

The availability of polyclonal and monoclonal antibodies directed against different HNE-protein adducts (involving cysteine, lysine, or histidine residues) allowed the formulation of immunodetection methods which are commercially available. For example, specific antibodies are used to detect HNE-histidine adducts in tissues or biological samples and HNE-modified tau protein has been associated with neurofibrillary tangles in Alzheimer's disease [217].

The reliability of immuno-based methods is mostly dependent on the specificity of the antibodies utilized, that may lead to differences between the available commercial kits. A fructosamine assay for the detection of ketamine formed via a nonenzymatic glycation reaction of serum protein, and the HPLC measurement of furosine, a specific product obtained after hydrolysis of epsilon-amino-fructose-lysine, are also alternative biomarkers [218, 219].

Specific AGEs, as pentosidine and carboxymethyl lysine, can be measured by HPLC [220, 221]. However, their use as biomarkers and the development of specific assays in clinical application are hampered by the structural heterogeneity of these products, due to different mechanisms of formation, and because few AGEs have been characterized.

In addition to the role as marker of oxidative stress, the clinical relevance of AGE is indicated by their pathogenic role in immune- and inflammatory-mediated diseases.

First of all, the role of the receptor for advanced glycation end products (RAGE)-NF-kB axis in neuroinflammation is in line with the nonenzymatic glycosylation theory of aging, suggesting a central role of the AGEs in the age-related cognitive decline [17]. Besides, the soluble receptor for advanced glycation end products (sRAGE) plays an important role in the pathogenesis of the acute respiratory distress syndrome [222].

On the other hand, Turk et al. suggested a role for AGE-immune complexes in the pathogenesis of atherosclerosis. Compared to healthy subjects, both diabetic and nondiabetic patients with coronary artery disease had a higher concentration of circulating immune complexes containing the AGE moiety as antigen, whereas only diabetics had higher anti-AGE antibodies [223]. Autoantibodies to IgG-AGE were detected in patients with rheumatoid arthritis, suggesting that glycation of IgG results in the generation of new immunogenic epitopes, potentially inducing circulating autoantibodies [224]. Therefore, AGEs could be one of the links between metabolic syndrome and immune activation.

3.3.2. Nitrotyrosine. 3-nitro-tyrosine (3-NO-Tyr) is the main product of tyrosine oxidation which may occur either within a polypeptide or in free tyrosine residues. This modification can be generated through several pathways that include the reaction with ROS and RNS like $ONOO^-$ and $NO_2^\bullet$ [225–227] (Figure 2). $NO^\bullet$ generated by NOS can react with $O_2^{\bullet-}$ to form $ONOO^-$ that, at acidic pH, is present as protonated form (ONOOH) which is believed to decompose into $HO^\bullet$ and $NO_2^\bullet$ to an extent of ~30% [10]. Generally, tyrosine oxidation is a two-step process with the formation of a tyrosine radical, generated by different oxidative steps, followed by the reaction with $NO_2^\bullet$. Accurate determination of 3-NO-Tyr in biological samples requires gas or liquid chromatographic techniques coupled to mass spectrometry [228–230], conditions that are not feasible for high throughput in clinical analysis. For a better determination, protein extracts from biological samples can be completely hydrolyzed before quantification of 3-NO-Tyr by chromatography. A pitfall in this technique is the possible nitration of tyrosine residues in the sample by the presence of nitrite and the acid conditions during protein precipitation and hydrolysis [231].

ELISA assay based on specific antibodies are also available, but their use is limited by the different affinity of antibodies for different nitrated proteins and the low sensitivity [232, 233]. 3-NO-Tyr has been described as a stable marker of oxidative/nitrative stress in inflammatory diseases [234, 235], but its utility as clinical biomarker is still questioned. Some studies showed that 3-NO-Tyr plasma levels are increased in several conditions, such as asthma, diabetes, and cardiovascular diseases, and reduced following therapeutic treatments [236, 237]. Moreover, an involvement of 3-NO-Tyr in age-related neurodegenerative diseases has been suggested [238, 239].

3.3.3. Advanced Oxidation Protein Products (AOPP). The reaction of proteins with chlorinated oxidants such as hypochlorous acid results in chlorination of amino acid residues and formation of 3-chloro-tyrosine (3-Cl-Tyr) and 3,5-dichloro-tyrosine as main products. These oxidation products are generally classified as advanced oxidation protein products (AOPP) (Figure 2) and include protein aggregates by disulphide bridges and/or tyrosine cross-linking. AOPP is a marker of oxidative stress that reflects the chronic kidney failure and has been identified as a marker of inflammation in many diseases [240–250]. Chloro-tyrosine, as well as 3-nitro-tyrosine, can be produced by reaction with ipochlorous acid and $ONOO^-$ both generated during inflammation, and it has been observed that AOPP may act as a mediator of the inflammation process and monocyte activation [240]. AOPP levels result as elevated in diseases such as diabetes, uremia, systemic sclerosis, atherosclerosis, and cardiovascular diseases and in patients with renal complications, increasing with the progression of chronic renal failure [241–244].

AOPP level can be measured by colorimetric tests using a chloramine standard or human serum albumin derivatives [245]. 3-Cl-Tyr is a highly specific biomarker that can be detected with very sensitive methods such as mass spectrometry [231, 246]. 3-Cl-Tyr has been detected in patients with atherosclerosis [247] and rheumatoid arthritis [248], in children with cystic fibrosis [249], and in the airways of preterm infants [250].

3.3.4. oxLDL. Low-density lipoproteins can undergo oxidative modification, and this has been correlated with atherosclerosis and cardiovascular diseases [251, 252].

The most common test makes use of specific antibodies that recognize selected modifications of LDL amino acidic residues (i.e., aldehyde-modified lysine residues or oxidized phospholipid-modified residues). However, the use of oxLDL as a biomarker of oxidative stress has been criticized because of the heterogeneity of oxidation products, the low specificity of the antibodies, and the different results obtained depending on the assay utilized [253, 254].

In addition, the clearance of oxLDL and the formation of immunocomplexes must be taken into account. Patients with ischemic stroke with intracranial atherosclerosis had a higher baseline level of oxLDL and a greater decline after a standardized fat meal compared to those that presented extracranial atherosclerosis, indicating an increase of the clearance of the oxLDL after meal [255]. An increase in the uptake of oxLDL has been observed also in macrophages from type 2 diabetes (T2D) patients [256], potentially inducing foam cell formation and atherosclerosis. oxLDL may also induce maturation of dendritic cells and regulate the shift from classical (M1) to alternative (M2) macrophage activation and from T helper 1 (Th1) to T helper 2 (Th2) response, suggesting that these could act as a bridge between innate and adaptive immunity, involved in plaque development [27]. The Th2-induced response could account to the presence of anti-oxLDL antibodies in subjects with T2D and impaired glucose tolerance [257], as well as to the anti-MDA-LDL IgGs found in serum of patients undergoing off-pump and on-pump coronary artery bypass grafting [258]. Therefore, oxLDL are not only a marker of oxidative stress but also a pathogenic factor whose values should be evaluated in the context of a global clinical examination.

3.3.5. Ischemia-Modified Albumin. Albumin, the most abundant protein in serum and other body fluids, is a carrier of many biomolecules. Albumin is susceptible to oxidation and carbonylation and may also act as an antioxidant system through the reversible oxidation of its cysteine residues. For this reason, it can be considered a general oxidative biomarker in several human diseases.

Table 3

Reversible cysteine modifications	*Methods*	*Limitations and confoundings*
S-glutathionylation GSH/GSSG SH	MS, ELISA, WB Spectrofotometric	For an accurate quantification, a specialized instrumentation is required
ROS-regulated transcription factors	*Methods*	*Limitations and confoundings*
Nrf-2, NF-kB	Immunological techniques, RT-PCR	
ROS-generating enzymes	*Methods*	*Limitations and confoundings*
NOX, MPO, XO, NOS	Immunological techniques, WB, PCR, RT-PCR, enzymatic	Antibody specificity Different percentages of leukocytes' populations
Antioxidant enzymes	*Methods*	*Limitations and confoundings*
SOD, CAT, GPX, GR	Immunological techniques WB, PCR, RT-PCR, enzymatic	Antibody specificity Different percentages of leukocytes' populations

CAT: catalase; GPX: glutathione peroxidase; GR: glutathione reductase; GSH: glutathione; MPO: Myeloperoxidase; MS: mass spectroscopy; NOS: nitric oxide synthases; NOX: NADPH oxidase; PCR: reverse-transcription polymerase chain reaction; SOD: superoxide dismutase; WB: Western blot; XO: xanthine oxidase.

Myocardial ischemia results in structural changes to the N-terminus of the serum albumin related to the production of ROS [259, 260]. These changes reduce the ability of albumin to bind transition metals, particularly cobalt cations, which can be detected by the albumin cobalt-binding (ACB) test [261–263]. Besides the N-terminal cobalt-binding site, albumin contains two additional sites that are negatively modulated by fatty acids binding to albumin. Therefore, it has been hypothesized that the release of fatty acids in myocardial ischemia is responsible for the lower cobalt-binding capability [264]. The ABC test indirectly detects Ischemia Modified Albumin (IMA) by measuring the decreased binding capacity of albumin for cobalt [265] and has been carried out by the Food and Drug Administration (FDA) to detect myocardial ischemia. Growing evidence suggest that IMA is not only specific for cardiac ischemia, but its elevated levels are also reported in patients with liver cirrhosis, pulmonary embolism, diabetes mellitus, cerebrovascular disease, and Alzheimer's disease [266–269]. Thus, measurements of IMA serum levels could be a new marker of oxidative imbalance. However, ACB test is sensitive to pH changes, altering the metal-binding capacity of the albumin, as well as temperature and time of sample storage. Analysis should be performed within 2 h or the serum should be separated and frozen [270, 271]. Recently, several immunoassays based on specific antibodies anti-IMA have been introduced in the market.

4. Redox Proteomic and Markers Based on Cysteine and Redox Enzymes

The powerful strategy offered by the mass-proteomic approach makes it now possible to reach high sensitivity and specificity in determining oxidative modifications of selected proteins. If fact, redox proteomic can provide information on both the identification of the oxidized protein and the extent of oxidative damage occurring at the protein level [272–274]. Proteins may become reversible oxidized in response to a redox signalling, but irreversible oxidative modifications are associated with disorders and pathologies [275, 276]. Thus, a profile of oxidative modification of plasma or tissue sample proteins is a promising approach that will help in clinical determination of several human diseases and pathological states [189, 276, 277]. This will also make the identification of novel biomarkers and therapeutic targets for different human diseases possible.

In particular, components whose deregulation can result in oxidative stress, such as the ROS-generating enzymes, and antioxidant defence systems, which change in response to increased redox stress, can be used to assess the redox state of the body or specific tissues and cells in health and disease.

In the context of redox proteomic, major players are cysteine residues (including the GSH system), antioxidant (SOD, CAT, and GPX), and ROS-generating enzymes, as well as the transcription factors involved in their regulation [278] (Table 3).

Surface-exposed cysteine residues are particularly sensitive to oxidation by ROS and RNS and are the most vulnerable among all amino acids [240].

Although the reactivity of thiol groups toward H_2O_2 is very low, the nucleophilicity and reactivity toward several ROS species, including $HO^{\bullet}$, HClO, $O_2^{\bullet-}$, and $NO^{\bullet}$, increase when the sulfur atom of the thiol group becomes deprotonated. Solvent exposure of the cysteine residue and the presence of neighbour polar residues exert a great influence on thiol group pKa. Thus, cysteine oxidation by ROS depends on the protein context and provides the basis for selective and specific modifications [279, 280].

The primary product of cysteine residue oxidation by H_2O_2 is the sulfenic acid (–SOH), whose stability and further reactivity may be influenced by the presence or availability of a proximal thiol group, resulting in the formation of a disulfide bond [281, 282] (Figure 3). Additionally, sulfenic acid may further react with H_2O_2 to produce sulfinic ($-SO_2H$) and sulfonic ($-SO_3H$) acids (Figure 3). Cysteine residues may also react with $HO^{\bullet}$ and $O_2^{\bullet-}$ species, resulting in the formation of a highly reactive radicalic sulfur atom ($RS^{\bullet}$), which can further react with another thiol residue generating a disulfide, while the reaction with $NO^{\bullet}$ produces a S-nitrosylated cysteine [283].

Oxidation of cysteine residues is reversible, with the exception of sulfinic and sulfonic acids products; it may be

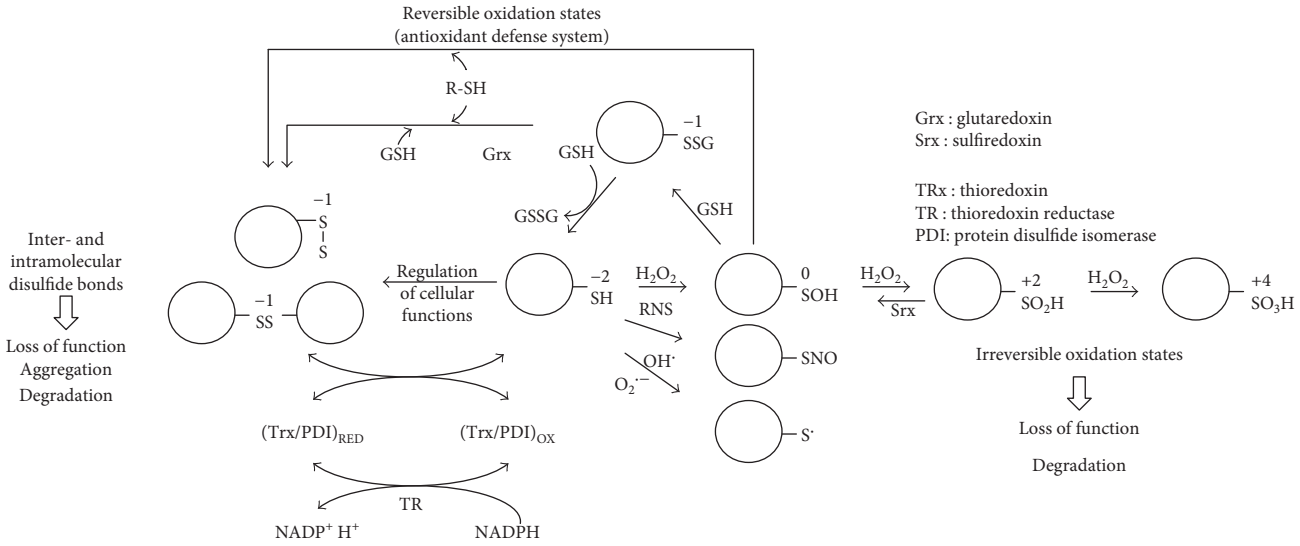

Figure 3: Reversible oxidation of protein cysteine residues. GSH: glutathione; H_2O_2: hydrogen peroxide; $O_2^{\bullet-}$: superoxide; RNS: reactive nitrogen species; $RS^{\bullet}$: sulfur atom; $-SO_2H$: sulfinic acid; $-SO_3H$: sulfonic acid; −SOH: sulfenic acid.

reversed to the thiol form by reaction with GSH and/or specific enzymatic activities (thioredoxins, glutaredoxins, and protein disulfide isomerases) [284–286] (Figure 3).

The reversible protein oxidation is an important feature for the antioxidant defence systems, which can efficiently help in reducing the intracellular levels of oxidized proteins, produced upon cell exposure to damaging agents, and prevent the accumulation of misfolded or self-aggregating proteins [190, 273, 287–289] (Figure 3).

Reversible protein modifications may be also an important feature for signalling pathways involving ROS and RNS through the chemical modification of selected substrate proteins. This provides the basis for several redox-regulated cellular processes and enzymatic functions which imply redox-dependent modifications [290–292]. So, protein oxidative modifications can be a consequence of oxidative or nitrosative stress as well as the reflection of redox-regulated processes [273, 293].

4.1. Protein Glutathionilation. Reversible protein-S-glutathionylation can occur either under physiological conditions, within redox signalling pathways, or as result of GSH antioxidant activity through the reduction of oxidized cysteine residues and the formation of mixed disulfide protein-glutathione (PSSG). Cysteine- (SOH-) glutathionilation may act as a protective mechanism preventing further irreversible oxidation to sulfinic or sulfonic acids [294]. Reduction of PSSG can take place spontaneously, when the GSH/GSSG ratio is high, or can be catalyzed by protein thiol-disulfide oxidoreductases, such as glutaredoxins, protein disulfide isomerases, thioredoxin, peroxiredoxins, and sulfiredoxins [295]. Recent advances in redox proteomic techniques have led to the identification of many S-glutathionylated proteins and their involvement in redox-regulated pathways. Reversible protein-S-glutathionylation in monocytes and macrophages has emerged as a new and important signalling paradigm, which provides a molecular basis for the well-established relationship between metabolic disorders, oxidative stress, and cardiovascular diseases [296].

Measurement of S-glutathionylation of functional important proteins is a promising biomarker. However, this is hampered by complexity in the methodologies (accessing tissue samples and procedural artefacts) which requires special care in sample handling and preparation [297]. A simpler approach is analyzing S-glutathionylation of proteins in circulating cells. Glutathionylation of haemoglobin has been proposed as a marker of oxidative stress, and an increase in protein modification has been reported in patients with diabetes, hyperlipidaemia, and renal failure [298, 299].

Although S-glutathionylation can be easily measured by Western blotting under nonreducing conditions, the use of more effective approaches is required for an accurate quantification. MS techniques are valid but require specialized instrumentation. In addition, ELISA tests using monoclonal anti-glutathione antibody have been developed [300–302].

4.2. Glutathione and Cysteine. GSH is a tripeptide representing the most abundant nonprotein thiol present in the cell, where its concentration can reach the millimolar range [303, 304]. GSH acts as an antioxidant defense system by its ability to scavenge ROS through the reversible oxidation to GSSG (Figure 3). GSSG can be enzymatically reduced to GSH by the activity of glutathione reductase (GR) and the reducing power of NADPH. Glutathione is mainly stored within the cytosol, where the ratio GSH/GSSG is ranging from 30 to 100 [305]. This ratio is ten times lower in the serum and in the endoplasmic reticulum and decreases in the presence of oxidative stress. Glutathione synthesis depends on the availability of cysteines, the rate-limiting precursor, and this makes its use as a marker of oxidative stress questionable. Besides, diurnal variation in GSH and cysteine has been reported [306]. However, several studies relate the GSH levels and GSH/GSSG ratio to pathological conditions [254]. The measurement of GSH, GSSG, and their ratio in

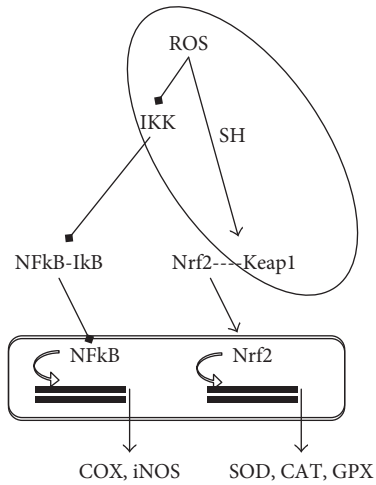

Figure 4: Cysteine-regulated gene expression. CAT catalase; COX: cyclooxygenase; GPX: glutathione peroxidase; IKK: Iκ kinases; iNOS: inducible nitric oxide synthase; Keap1: Kelch-like ECH-associating protein 1; Nfr2: nuclear factor-erythroid 2-related factor 2; NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells; ROS: reactive oxygen species; SH: thiol; SOD: superoxide dismutase.

blood has been considered an index of the redox status in the whole-organism and a useful marker of diseases in humans [307, 308]. Several methods have been used to determine the GSH in biological samples (spectrophotometry, HPLC, capillary electrophoresis, nuclear magnetic resonance, and mass spectrometry) [307]. However, GSH and its oxidate form GSSG do not represent powerful biomarkers of oxidative stress because of some methodological artifacts. For instance, sample acidification for protein precipitation leads to an increase in GSSG levels [308].

4.3. Nrf-2 and NF-kB. As mentioned above, oxidation of selected cysteine residues present in specific proteins may result in the regulation of cellular response to oxidative stress. This is the case for Nrf-2, a conserved transcription factor that is a master regulator of the antioxidant response system controlling the expression of more than 250 genes. Nrf-2 is normally sequestered into the cytoplasm complexed to the protein Keap1 (Kelch-like ECH-associating protein 1), which facilitates its polyubiquitination and proteasome-mediated degradation [309]. Keap1 contains specific cysteine residues sensitive to oxidation in the presence of oxidants or other electrophiles (Figure 4). Thus, Keap1 functions as a specific sensor of stress that upon oxidation, and resulting conformational change, releases Nrf-2 allowing its translocation into the nucleus.

Nrf-2 promotes the transcriptional activation of a specific set of target genes containing the antioxidant response elements (AREs) in their promoter regions and encoding antioxidant and detoxifying enzymes (i.e., glutathione S-transferase, glutathione synthetase, heme oxygenase 1, and NAPH-oxidoreductase) (Figure 4). Thus, Nrf-2 is related to the cellular defence against ROS and it has been observed that its activity declines with age as well as with degenerative disorders [310].

On the other hand, an increased Nrf-2 activity has been observed in transformed cells [311], where it provides a reduced sensitivity both to the large amounts of ROS generated during the active proliferation and to chemotherapeutic drugs, whose enzymatic elimination requires enhanced levels of NAPDH. For these reasons, Nrf-2 can be considered a valid biomarker and its levels in tumour samples, quantified by immunological methods or by RT-PCR, may have a clinical significance. Recently, the determination of Nrf-2 levels, in combination with measuring high-mobility group box-1 (HMGB1) expression, might represent a useful tool in the early detection of post-trauma complications [312].

Whereas Nrf2 has a primary role in antioxidant enzymes gene expression, NF-kB is involved in the transcription of ROS-generating and inflammatory enzymes (Figure 4). As observed for Nrf2, some cysteine residues are involved in the translocation of NF-kB to the nucleus (Figure 4). In particular, cysteine 179 of Iκ kinases (IKK) is a target of the S-glutathionylation-induced inactivation and glutaredoxin reverses this effect [313]. Furthermore, electrophilic modifications of cysteine 179 of IKK inhibit NF-kB activation and have been suggested as one of the mechanisms involved in the anti-inflammatory and COX-inhibitory effects of nutraceuticals [314, 315]. Similarly, antioxidants with catechol and electrophilic moieties induce the Nrf2-mediated gene expression of antioxidant enzymes acting as pro-oxidants rather than antioxidants [316, 317].

4.4. Enzymes. ROS-generating enzymes are involved in several cell functions and their alteration may result in imbalanced redox status (Figure 5).

The established role in diseases of XO [318] and NOX [319, 320] suggested their pharmacological inhibition in the prevention and treatment of pathologies related to oxidative stress.

Some ROS-generating enzymes can be found in the circulation and thus can be used as markers of oxidative stress, such as NOS and NOX (Figure 5) involved in oxidative burst.

It has been shown that high levels in the circulations of MPO, a heme peroxidase abundant in granules of human inflammatory cells, which catalyzes the conversion of H_2O_2 to HClO with the production of ROS (Figure 5), are associated with cardiovascular disease [321], chronic obstructive pulmonary disease [322], and Alzheimer's disease [323].

Oxidant species derived from MPO lead to the production of specific oxidation products, such as 3-Cl-Tyr. This can be used as biomarker in several diseases [324], as above described, and its levels correlate with MPO. However, expensive equipment are required to detect the levels of MPO-dependent specific biomarkers and this represents a limitation in their use. Moreover, the concentration of these biomarkers in biological samples is low, which complicates accurate measurement.

XO catalyzes the oxidation of hypoxanthine and xanthine to UA in the terminal steps of purine nucleotide metabolism [325], which also leads to the production of $O_2^{\bullet-}$ [326] (Figure 5). Given that XO produces ROS in stoichiometric quantities along with UA, it represents one of the major sources of free oxygen radicals in human physiology. Upregulation of XO activity may lead to an increase in UA serum

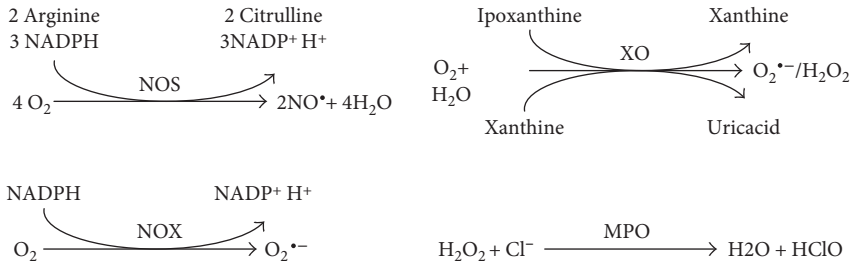

Figure 5: ROS generating enzymes. H_2O_2: hydrogen peroxide; HClO: hypochlorous acid; MPO: myeloperoxidase; NOS: NO synthase; NOX: NADPH oxidase; $O_2^{\bullet-}$: superoxide; XO: xanthine oxidase.

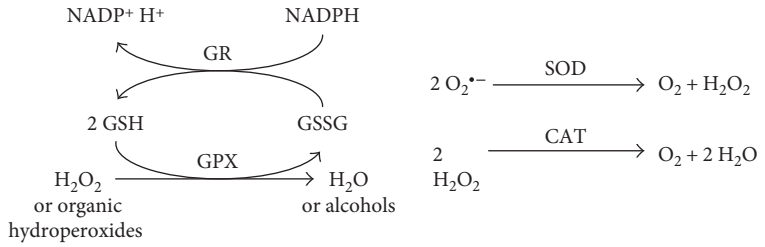

Figure 6: Antioxidant enzymes. CAT: catalase; GPX: glutathione peroxidase; GR: glutathione reductase; H_2O_2: hydrogen peroxide; $O_2^{\bullet-}$: superoxide; SOD: superoxide dismutase.

levels, oxidative stress, and endothelial dysfunction [327–329]. XO exists in two interconvertible forms, XO (that oxidizes xanthine to UA using oxygen as the electron acceptor and produces superoxide or H_2O_2) and xanthine dehydrogenase (XDH) (that carries out the same reaction but uses NAD^+ and generates NADH). XDH is the predominant form in well-oxygenized tissue [330], but it can be converted to XO under various conditions [331, 332]. Inflammatory or hypoxic conditions promote XDH expression in tissues and vascular endothelial cells and stimulate XDH release into the circulation [333]. Once in the circulation, XDH is quickly converted, by reversible oxidation of the sulfhydryl residue or by irreversible proteolysis, into XO which binds to the endothelial surface, resulting in amplified XO-derived ROS formation [334]. This XO-induced oxidative stress has been detected in renal and cardiovascular diseases, such as heart failure, chronic obstructive pulmonary disease, pulmonary hypertension, sickle cell disease, and diabetes [334]. An increase in XO activity has been reported in patients with heart failure [326, 335], whereas XO activity and its plasma levels are raised in presence of inflammatory agents and interferon [336] and seems to play a key role in ischemia-reperfusion injury [337].

As described for MPO, an indicator of the enzyme activity in vivo could be the detection of a metabolite or a reaction product. Serum levels of UA may reflect XO activity, but they are also dependent on dietary intake, and purine metabolism, and renal filtration and reabsorption, as well as endothelia dysfunctions. Higher UA levels are associated with metabolic, cardiovascular, and renal abnormalities, and UA has been recently proposed as a biomarker and therapeutic target in diabetes [338–340]. UA is a powerful antioxidant in plasma and can scavenge $O_2^{\bullet-}$ and $HO^{\bullet}$, and allantoin is its oxidative product of which formation is independent of changes in UA levels [341–343]. This makes allantoin a promising biomarker of oxidative status, considering also its stability regardless of the storage or sample preparation, but its quantitative determination requires specific instrumental techniques as liquid/gas chromatography and mass spectrometry [344–346].

The most important antioxidant enzymes are SOD, CAT, and glutathione-dependent enzymes, such as GPX, GR, and glutathione transferases (GSTs) (Figure 6).

SODs are a family of enzymes catalyzing dismutation of superoxide into oxygen and H_2O_2. There are three isoforms of SOD, with a different cellular localization and metal cofactor: homodimeric Cu/Zn-SOD localized in the cytosol and in the mitochondrial intermembrane space, homotetrameric Cu/Zn-SOD with an extracellular distribution, and homotetrameric Mn-SOD localized in the mitochondria [347]. SOD acts also as pro-oxidant producing H_2O_2; therefore, other antioxidant enzymes such as CAT and GPX are required and an imbalance in their ratio may be dangerous.

SOD activity can be measured analyzing the inhibition in the rate of reduction of a tetrazolium salt by $O_2^{\bullet-}$ generated through a xanthine/XO enzymatic system [348, 349].

CAT, which catalyzes the conversion of H_2O_2 into water and oxygen, is a homotetrameric protein containing four iron heme and largely located in the peroxisomes. CAT activity can be measured by several colorimetric/spectrophotometric assays [349, 350].

GSH redox cycle is regulated by GPX and GX. GPXs are a family of selenium-dependent isozymes that catalyze the reduction of H_2O_2 or organic hydroperoxides to water and alcohols through the oxidation of GSH to GSSG. GR then

reconverts GSSG to GSH using the reducing power of NADPH [351]. GPX activity can be measured using cumene hydroperoxide and GSH as substrates in a coupled reaction with GR [352]. The GSSG formed after reduction of hydroperoxide is recycled to its reduced state by GR in the presence of NADPH. The oxidation of NADPH is accompanied by a decrease in absorbance at 340 nm proportional to GPX activity. GR activity can be measured in a similar manner using GSSG and NADPH as substrates [353].

Differently from ROS-generating enzymes, conflicting results came from human studies that evaluated the relationship between diseases or ageing and antioxidant enzymes. Despite meta-analyses suggesting that polymorphisms of antioxidant enzymes are associated with T2D [354] and hypertension [355], decreased or increased activities (or levels) have been reported for SOD, catalase, GPX, and/or GR in these diseases [356–367]. Activity of SOD or CAT was significantly higher in elderly hypertensives [356] and T2D [360, 361, 368] when compared with healthy controls. Increased SOD activity has been reported also in coronary artery disease patients [369] and in women with the polycystic ovary syndrome [370]. In patients with Crohn's disease, SOD and GPX increase during the active phase and return to normal during the remission phase [371]. It has been suggested that the increase in antioxidant enzymes may represent a compensatory upregulation in response to increased oxidative stress [361, 368]. Results of Karaouzene et al. suggest that this response depends on age [372]. Erythrocyte SOD and CAT activities were enhanced in obese young patients but reduced in obese older men [372]. The ARE/Nrf2 pathway is the major player in the induction of the expression of antioxidant genes [309]. However, although phytochemicals contained in fruits and vegetables are known to induce Keap1/Nrf2 system [373] in a meta-analysis [374] of randomized controlled trials, no significant differences were observed between fruit or vegetable juices and placebo in SOD and CAT, despite the reduction of MDA.

In order to understand the contrasting results in human studies, some methodological considerations must be made. Conventional methods for measuring enzymes are enzyme activity, protein content (Western blots and immunological techniques), or gene expression (reverse-transcription polymerase chain reaction (RT-PCR)) (Table 3) [129]. First of all, it must be taken into account that different samples have different antioxidant content. In a meta-analysis, decreased activities of SOD and GPX were observed in plasma/serum of postmenopausal women with osteoporosis, but the activities of SOD in erythrocytes and of CAT in plasma/serum were not statistically different from the control group [375]. Concerning the measure of cellular enzymes, it must be considered that processing and cryopreservation procedures could affect peripheral blood mononuclear cell (PBMC) gene expression [376, 377]. In addition, PBMC exclude from the analysis the neutrophils that are the major component of the full blood count [378], reducing the clinical relevance of this sample compared to whole blood RNA. On the other hand, the different cell types present in blood have a different content of enzymes. In whole-blood iNOS, RNA was expressed predominantly in monocytes [379]. Although the presence of MPO in lymphocytes has been recently reported, it is very low compared to neutrophils/monocytes [380]. Concerning antioxidant enzymes, neutrophils have higher levels of SOD and catalase transcripts compared to monocytes [381]. On the other hand, GSH content and GPX transcript and activity are higher in monocytes [381]. In this context, results from meta-analysis document that neutrophil-to-lymphocyte ratio [382–387] and lymphocyte-to-monocyte ratio [388, 389] were related to clinical oncological outcomes in cancer patients. Also, coronary artery disease is associated with altered ratio of leukocytes [390], the expansion of monocytes, and the reduction of the CD4/CD8 T cell ratio, and B cell lymphopenia can be observed in end-stage renal disease [391]. Furthermore, also in heathy subjects, the normal ranges of the different leukocyte populations are very large [392]. Probably, the use of cell marker coding genes (CD4, CD8, CD14, etc.) as housekeeping genes could normalize the results for the physiologically or pathologically different content of cells between subjects [378]. This approach could also help in conditions, such as hyperglycemia, that can influence the expression of housekeeping genes [393].

5. Measuring the Nonenzymatic Antioxidant Capacity in Body Fluids

The nonenzymatic antioxidant capacity (NEAC), also named total antioxidant capacity (TAC), is defined as the moles of oxidants neutralized by one liter of body fluids [278, 394–396]. In plasma, nonenzymatic antioxidants include endogenous (e.g., UA, bilirubin, and thiols) and nutritional (e.g., tocopherols, ascorbic acid, carotenoids, and phenolics) compounds [278, 394]. Various assays for NEAC [129, 397–412] measure either their radical scavenging or reducing capacity. Reaction mechanisms include hydrogen atom transfer (HAT) and single electron transfer (SET) (Table 4). The latter reports on antioxidants' reductive capacity, including its metal reducing power, and could be considered an "indirect assay," whereas the former is a "direct assay" (competitive) in which the inhibition of the oxidation of an indicator substance is determined as a measure of the antioxidant capacity [406, 407]. The most common HAT methods are oxygen radical antioxidant capacity (ORAC) and the total radical-trapping antioxidant parameter (TRAP), performed in aqueous solutions with 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) as a thermolabile stoichiometric and water-soluble azo-radical generator (Table 4).

The Crocin bleaching assay can be performed under both hydrophilic and lipophilic conditions by using AAPH or 2,2′-azobis 2,4-dimethylnaleronitrile (AMVN), which is AAPH's lipophilic equivalent. Aldini et al. [401] monitored the oxidation of the lipid compartment of plasma by using 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeO-AMVN), as lipid soluble radical initiator and C11-BODIPY$^{581/591}$ as lipophilic fluorescence probe. The MeO-AMVN-C11-BODIPY$^{581/591}$-based total antioxidant performance (TAP) assay was reported to be sensitive to plasma antioxidants localized in both the lipophilic and hydrophilic compartments [400]. In all HAT methods, ROO$^{\bullet}$ reacts with the target

Table 4: Common used methods for NEAC measurements.

Method	Reaction and quantification	Limitations and confoundings
HAT ORAC	AAPH—induced: R-phycoerytherin (red) or fluorescein (green) fluorescence decay Competitive reaction kinetic, AUC	Lipophilic antioxidants not included Proteins
HAT TRAP	AAPH—induced: R-phycoerytherin fluorescence decay (red) DCFH ➔ DCF fluorescence increase (green) Competitive reaction kinetic, lag phase	Lipophilic antioxidants not included Not all the antioxidants give a lag phase Self-propagation of DCF radicals
HAT Crocin bleaching	AAPH- or AMVN-induced absorbance decay (450 nm) Competitive reaction kinetic, Stern-Volmer-like relation	Bilirubin and carotenoids that absorb at the wavelength of determination
HAT TAP	MeO-AMVN induced C11-BODIPY fluorescence increase (green) Competitive reaction kinetic, AUC	
TAS	Fenton reaction-induced dianisidyl radical absorbance increase (444 nm) Competitive, endpoint, TEAC	
SET (Randox)	Fenton reaction-induced ABTS radical formation (734 nm) Competitive reaction, endpoint, TEAC	
SET $ABTS^{\bullet+}$	Absorbance decay (734 nm) Noncompetitive, endpoint, TEAC	
SET $DPPH^{\bullet}$	Absorbance decay (515 nm) Noncompetitive, endpoint, EC50	Carotenoids that absorb at the wavelength of determination
SET FRAP	Absorbance increase (593 nm) Noncompetitive, endpoint	SH not included Biliverdin absorb at the wavelength of determination
SET CUPRAC	Neocuproine absorbance increase (450 nm). Noncompetitive, endpoint	Bilirubin and carotenoids that absorb at the wavelength of determination

AAPH: 2,2′-azobis(2-methylpropionamidine) dihydrochloride; ABTS: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); AMVN: 2,2′-azobis 2,4-dimethylnaleronitrile; AUC: area under the curve; CUPRAC: copper-reducing assay; DCFH: 2′,7′-dichlorodihydrofluorescein; DPPH: 2,2-diphenyl-1-picrylhydrazyl; EC50: efficient concentration (EC), the amount of antioxidant necessary to decrease by 50%; FRAP: ferric reducing antioxidant power; HA: T hydrogen atom transfer; MeO-AMVN: 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile); NEAC: nonenzymatic antioxidant capacity; ORAC: oxygen radical antioxidant capacity; SET: single electron transfer; SH: thiols; TAC: total antioxidant capacity; TAP: total antioxidant performance; TAS: total antioxidant status; TEAC: Trolox equivalent antioxidant capacity; TRAP: total radical-trapping antioxidant parameter.

compound resulting in changes of fluorescence or absorbance of probe (Table 4).

Area under the curve (AUC), lag phase, or Stern-Volmer-like relation are used in order to measure the competition reaction and the standard antioxidant Trolox is used as reference (Table 4). NEAC values are reported as Trolox equivalents (TEAC) also in the total antioxidant status (TAS) assay and in the competitive (Randox) SET-based 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assay, both based on the production of $HO^{\bullet}$ via Fenton reaction [399]. In other SET assays, using the stable radical cation $ABTS^{\bullet+}$ or 2,2-diphenyl-1-picrylhydrazyl (DPPH), the target compound extracts an electron from the antioxidant and changes color (Table 4). In these assays, it is assumed that antioxidant activity is equal to reducing capacity [406]. Other SET methods measure the reducing power of antioxidants through redox reaction with iron (ferric reducing antioxidant potential (FRAP)) or copper (cupric reducing antioxidant capacity (CUPRAC)) (Table 4). The latter has been applied to both lipophilic and hydrophilic fractions of serum [398]. However, as for Crocin bleaching assay, bilirubin and carotenoids that absorb at the wavelength of determination could interfere with the results (Table 4). Similarly, the oxidation product of bilirubin (biliverdin) absorbs at the wavelength of determination of FRAP method (Table 4). Although NEAC assays present the advantage of integration of the individual antioxidant actions of different compounds and their additive, synergistic, or antagonistic interactions, many limitations have been pointed out previously [129, 403–407] (Table 4). Different NEAC assays can give different results, both in disease states [396] and after dietary supplementation with antioxidant-rich plant foods and beverages [394, 412, 413]. In a meta-analysis, Lettieri et al. [394] reported that TRAP, ORAC, and FRAP, but not TEAC, displayed an increase in plasma NEAC in both acute and chronic studies. From that, the authors [394] suggested that FRAP could be more sensitive than the TEAC assay within the SET methods to assess plasma NEAC. Accordingly, Carrión-García et al. [414] found a statistically significant positive correlation between plasma FRAP and dietary FRAP, either derived

from the food frequency questionnaire (FFQ) and/or from a 24-hour recall (24-HR), whereas plasma ORAC without proteins, but not plasma ORAC, was related with 24-HR-based dietary ORAC, suggesting that proteins rather than dietary antioxidants have a primary role in plasma antioxidant defences. Despite FRAP appearing to be the more sensitive method to evaluate the effects of antioxidant-rich foods on NEAC, it must be taken into account that reduced iron is the major player in the Fenton reaction. Therefore, an increase in the iron reducing power could be more likely detrimental than beneficial in conditions of high levels of iron and low levels of antioxidant enzymes [364, 396, 415–420]. On the other hand, an increase in antioxidant enzymes as adaptive response to oxidative stress has been observed in T2D [358, 359, 363, 368]. Simultaneously with increased MDA levels, significantly higher activities or levels of SOD and/or CAT were found [358, 359, 363, 368]. Some studies reported unchanged or decreased CAT and/or GPX and elevated SOD and lipoperoxidation markers in T2D [360, 361, 412] and CVD [369, 421]. The balance between SOD and CAT and/or GPX dictates H_2O_2 levels that may potentially react with reduced metals. As previously pointed out, in NEAC assays the contribution of the antioxidant enzymes is neglected [405, 406]. Therefore, the lower total antioxidant status in these cases must be interpreted with caution [421, 422].

Despite a correspondence between the effect on F2-IsoPs (the golden standard of oxidative stress) and NEAC has been reported in 67% (14/21) of the interventions with foods and in 77% (10/13) of the interventions with galenics [413] of human studies in a systematic review, in the majority of the cases the correspondence was the lack of change for both biomarkers, whereas increases in NEAC and decreases in F2-IsoPs were observed only in 9.5% (2/21) of interventions with foods and 30.7% (4/13) of interventions with supplements [413]. Furthermore, despite gas chromatography mass spectrometry or liquid chromatography mass spectrometry techniques giving a more reliable and precise measure of F2-IsoPs, in the majority of these studies (5/6), enzyme-linked immunosorbent assay-based methods were used [413]. Last, but not least, in one of these studies [423], the increase in NEAC and the decrease in F2-IsoPs were not associated with lipid and glucose metabolism markers, nor with renal and liver functionality markers in uremic patients after 4 weeks of supplementation with Emblica officinalis extract, suggesting a low clinical relevance of NEAC in certain conditions. In this context, the major bias of all methods is that, despite hyperuricemia being detrimental and associated with CVD [424–426], UA is the major contributor of NEAC measured in plasma (60–80%), saliva (70%), and urine (75%) [395]. In case-control studies, there was an accordance between UA concentration and NEAC, as well as between salivary or urinary NEAC and plasma or serum NEAC [395]. On the other hand, only in 44% of the interventions with antioxidant foods, beverages or supplements urinary NEAC was related to UA, probably due to the excretion of phenolic metabolites.

In order to avoid the UA interference, methods for UA independent NEAC have been proposed in both plasma and urine [427–430]. In particular, the consumption of 500 g of strawberries daily for 9 days had no effect on circulating phenolics and plasma NEAC, whereas it increased UA-independent NEAC and urinary metabolites of polyphenols [431]. Furthermore, it has been suggested that urinary UA-independent NEAC normalized for creatinine could provide more reliable information about the antioxidant status in children and adults with Down syndrome [429].

Although UA-independent NEAC could be a good approach also for salivary NEAC, it has been observed that salivary NEAC was affected by emotional and psychological factors [432]. The latter could induce hyperactive sympathetic nervous system and the activation of platelets [433], potentially changing the plasma NEAC. In this context, it has been suggested that plasma and not serum should be preferred for NEAC measurement, in order to avoid ROS generation by platelets during processing (aggregation) [434]. However, platelets' activation can occur during the time course of NEAC methods in plasma samples and, alternatively, vigorous vortexing produces platelets microparticles further confounding the results.

In fact, sample type, collection, processing, and methodological limitation must be taken into account when measuring NEAC. Despite the fact that the use of a refrigerated microcentrifuge to rapidly prepare plasma could avoid any thermal stress and instability of antioxidants in biological samples [434], recent results indicate that centrifugation at room temperature is the preferred option for many applications, giving lower microparticles and less hemolysis in plasma [435]. Hemolysis could bias many NEAC methods, and the presence of platelets and microparticles in the reaction mixture could affect the results. Considering that microparticle count is lower in serum compared with plasma after centrifugation at both room temperature ($1.73 \times 10\text{^}7$/ml versus $3.72 \times 10\text{^}7$/ml) and 4°C ($1.33 \times 10\text{^}7$/ml versus $7.4 \times 10\text{^}7$/ml) [435], probably serum and not plasma could be the better sample for NEAC evaluation.

On the other hand, although NEAC of saliva or urine has led to increasing interest, due to simple and noninvasive collections, many factors could give spurious results. In particular, blood contamination, periodontal diseases, bacterial counts and flow rate must be evaluated in order to avoid misinterpretation of the results of salivary NEAC and normalize for dilution [395, 436]. Also, urinary samples require normalization for dilution [395]. Despite results are normalized for the creatinine excretion, age, sex, muscle mass, renal diseases, and diet all have an influence on creatinine excretion [395].

From the mentioned above limitations and potential confounding, it appears that a clear association between increase in NEAC and health benefit is difficult to evaluate [405, 406]. Therefore, as previously suggested, each study requires a careful design of the experimental protocol and caution should be taken in the interpretation of results.

6. Conclusion

A clinically useful biomarker, besides being correctly measured, must be diagnostic, have prognostic value, and correlate with the disease degree. It must also be reasonably

stable, present in an easily accessible specimen, and its measurement should be cost-effective.

In order to evaluate the redox status in particular conditions (smoking habit, disease states), ex vivo free-radical production and oxidative stress in body fluids are measured. These methods are used also in human intervention studies to associate the levels of ingested antioxidants (by foods or supplements) with improvement of the body antioxidant status. Despite the fact that it has been suggested that nutraceuticals are capable of improving health, significant methodological bias must be taken into account in the interpretation of data from the measurement of reactive species in leukocytes and platelets by flow cytometry, from the evaluation of markers based on ROS-induced modifications, from the assay of the enzymatic players of redox status, and from the measurement of the total antioxidant capacity of human body fluids.

It has been suggested that the bias of each method could be overcome by the evaluation of oxidative stress by using more than one criterion [129, 404]. In this context, indexes of redox status have been proposed [437, 438].

The OXY-SCORE [437] was computed by subtracting the protection score (GSH, alpha- and gamma-tocopherol levels, and antioxidant capacity) from the damage score (plasma free and total malondialdehyde, GSSG/GSH ratio, and urine F2-IsoPs). The oxidative-INDEX [438] was calculated by subtracting the OXY (the antioxidant capacity measured with the OXY adsorbent test) standardized variable from the ROM (the reactive oxygen metabolites measured with the d-ROM) standardized variable.

These scores are related to CVD, age, gender, and smoking habit [437–442]. The oxidative-INDEX has been successfully used also in case-control studies (liver diseases and cancers) [443–445] and in a human intervention study with antioxidant [446].

More recently, a multiple factor analysis (MFA) that allows for simultaneous analysis of multiple parameters, classified according to their physiological meaning in athletes following strenuous endurance exercise, was applied [447]. This integrative approach reveals a close relationship between the oxidative index, the inflammatory IL-8, and the cardiac marker N-terminal pro-B-type natriuretic peptide (NT-proBNP). Athletes that showed a higher improvement of the oxidative index after the race, presented small changes in NT-proBNP and IL-8 levels, whereas subjects with minimal variation in the oxidative index had a marked postrace increase in NT-proBNP and IL-8 concentrations.

On the other hand, in some diseases, the choice of the markers that must be considered in the global index should dictate the clinical relevance in the subjects selected. Condezo-Hoyos et al. [448] measured an array of oxidative stress biomarkers (SH, GSH, UA, ORAC, MDA-bound protein, protein carbonyls, AOPP, 3-nitrotyrosine, CAT, XO, and MPO) in patients with chronic venous insufficiency (CVI) and used for the OXyVen index calculation the normalized and standardized plasma parameters which showed a significant statistical difference between CVI patients and controls (SH, MDA-bound protein, protein carbonyls, and CAT activity).

In conclusion, the clinical significance of biomarkers of oxidative stress in humans must come from a critical analysis of the markers that should be dictated by the study aim and design and should give overall an index of redox status in particular conditions.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Lycium barbarum Reduces Abdominal Fat and Improves Lipid Profile and Antioxidant Status in Patients with Metabolic Syndrome

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Natural antioxidants present in fruits have attracted considerable interest due to their presumed safety and potential nutritional value. Even though antioxidant activities of many fruits have been reported, the effects of phytochemicals of goji berry (GB) in patients with metabolic syndrome have not been investigated. In this study, we examined anthropometric and biochemical parameters in patients with metabolic syndrome after the consumption of GB. The patients were divided into two groups, control (C) and supplemented (S), and followed up for 45 days. Participants were individually instructed to carry out a healthy diet, but additionally, an inclusion of 14 g of the natural form of goji berry in the diet during 45 days for the S group was proposed. After 45 days of study, a significant reduction in transaminases as well as an improvement in lipid profile in the S group was observed. Likewise, a significant reduction in the waist circumference of the S group was observed when compared with that of the C group, and increased glutathione and catalase levels associated with a reduction of lipid peroxidation. These results suggest that this is an effective dietary supplement for the prevention of cardiovascular diseases in individuals with metabolic syndrome.

1. Introduction

Metabolic syndrome (MS) consists of different risk factors for cardiovascular disease (CVD) and diabetes mellitus type 2 (DM2) [1], and it includes a cluster of metabolic abnormalities such as abdominal obesity, dyslipidemia, hyperglycemia, and hypertension, leading to an increase in the levels of oxidative stress, concomitantly reducing antioxidant defenses [2, 3]. It is one of the main clinical challenges in public health, being considered a group of risk factors for cardiovascular disease, due to the accumulation of abdominal fat and the increase in inflammatory mediators and oxidative stress [3].

Oxidative stress has been implicated in the development and progression of many diseases such as atherosclerosis, inflammation, cancer, neurodegenerative diseases, and diabetes [4–6]. Redox homeostasis, being a metabolic equilibrium between reduction and oxidation, is important in maintaining normal metabolism by ensuring proper response from the cells to either endogenous or exogenous stimuli. Energy harvesting through cellular redox process releases by-products as reactive species: oxygen (ROS) and nitrogen (RNS). These reactive species are crucial for cell signaling. Overwhelming levels and dysregulation of the reactive species, however, disrupt the delicate balance [7]. The shift towards the oxidized state

leads to oxidative stress which can be defined as an imbalance between endogenous antioxidant defense mechanisms and the production of ROS, which at high levels can cause cell injury and damage through modifications of proteins, lipids, and DNA [8, 9].

In order to reduce the deleterious effects of oxidative stress, several antioxidant protective networks and signaling pathways are operative in cells [10]. The most important enzymatic antioxidant system is composed of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), which protect the cells against damage caused by the radical superoxide and hydrogen peroxide. In addition to the enzymatic system, the cells have a nonenzymatic system, especially glutathione [8, 9].

Previous studies have demonstrated a close relationship between the complications associated with MS and subcutaneous adipose tissue, as well as the characteristics of the visceral adipose tissue. When the adipocyte homeostasis is altered due to excessive calorie intake, sedentarism, or genetic variants, among others, several inflammatory adipose tissue-derived cytokines are released, such as interleukin 1 beta (IL-1β), interleukin 18 (IL-18), interleukin 6 (IL-6), and tumor necrosis factor alpha (TNF-α), as well as induction of mononuclear leukocytes (lymphocytes and monocytes) [11]. Such cytokines constitute a well-stablished link between insulin resistance and endothelial dysfunction, a precursor of atherosclerosis, another hallmark of MS [12].

Epidemiological studies have found that consumption of fruits and vegetables has attracted growing interest because of their significant role in reducing the risk of cardiovascular diseases and other chronic diseases [13, 14]. Several studies demonstrated that medicinal plants and fruits are a rich source of antioxidant compounds such as phenolics, flavonoids, quinones, vitamins, and alkaloids, which can decrease the incidence of oxidative stress and associated diseases [15–17]. Recent studies have demonstrated the effects of polyphenolic compounds in improving clinical factors associated with MS. Epidemiological evidence shows that the consumption of food rich in polyphenolic compounds in Asian populations reduces cardiovascular risk factors. However, there are limited studies associating the consumption of polyphenolic compounds in western populations [16].

A negative correlation has been shown between MS and polyphenolic compound ingestion and vitamins A, C, and E [17]. The addition of flavonoids on a diet could be an effective strategy for MS prevention with possible improvements in blood pressure, systemic inflammation, and oxidative stress [18].

Natural antioxidants that are presented in fruits have attracted considerable interest because of their presumed safety and potential nutritional and therapeutic value. The increased interest in natural antioxidants has led to the antioxidant evaluation of many species of fruits [14].

Among the fruits with the highest amount of antioxidant substances is the *Lycium barbarum* also named goji berry (GB). *Lycium barbarum*, a traditional Chinese medicine plant, is a small red fruit widely consumed in China due to its benefits to vision, kidney, liver, and diseases such as diabetes mellitus and obesity [19]. It is considered the richest natural source of antioxidants vitamin C, flavonoids, and carotenoids especially zeaxanthin, quercetin, and rutin [20–22].

A number of preclinical studies and a few clinical studies on the pharmacological activities and possible mechanisms of GB have been reported in the literature. GB exhibits a wide array of therapeutic/medicinal effects on aging, fatigue, cancer, colitis, stroke, diabetes, Alzheimer's disease, hepatoprotection, immunomodulation, and glaucoma in different animal models [23–26].

Although the antioxidant benefits of GB have already been highlighted in several in vitro and in vivo studies, no previous study has shown the effect of GB supplementation *in natura* on MS patients. In this study, we hypothesize that the administration of GB in patients with MS could positively influence glucose homeostasis, lipid profile, hepatic markers, and biomarkers of inflammation and oxidative stress.

2. Material and Methods

2.1. Participants. This study was approved by the University of Alto Vale do Rio do Peixe, and free informed consent of all participants was obtained (CAAE 1,304,016/2015). The recruitment has been made during individualized clinical evaluation in the Basic Health Unit of Peritiba, Santa Catarina, Brazil. Patients were selected according to initial screening results based on biochemical and anthropometric measurements. All participants were included as MS patients when possessing at least 3 of the following characteristics: abdominal obesity (waist circumference ≥ 90 cm in men and ≥ 80 cm in women); hypertriglyceridemia (triglycerides (TG) > 150 mg/dl); low high-density lipoprotein (HDL-c < 40 mg/dl in men and < 50 mg/dl in women); high low-density lipoprotein (LDL-C > 160 mg/dl); increased systolic blood pressure (SBP) ≥ 130 mmHg and diastolic blood pressure (DBP) ≥ 85 mmHg; and glycemia > 100 mg/dl in men or women in adulthood or elderly stage between the ages of 32 and 76. Individuals using antidiabetic, antihypertensive, and lipid-lowering medications were considered with fasting glucose, high blood pressure, and dyslipidemia, respectively. Exclusion criteria included not being part of individualized care, not being diagnosed with MS, not following the treatment, and showing any hypersensitivity to GB usage. The study consisted of 50 patients, randomly divided into two groups, referred to as the control group (C) and supplemented group (S).

2.2. GB Intake Intervention. GB used in the study were purchased from Ningxia Toyo Trade Co., Ltd., China. Portions of GB were provided in a daily fractionated portions. All participants were individually oriented to carry out a healthy diet, based on recommendations according to the IV Brazilian Guidelines on Dyslipidemia and Atherosclerosis Prevention [27]. The guidelines were as follows: hold three meals and two snacks throughout the day and do not skip meals; include daily six portions of the group of cereals, tubers, and roots, preferring whole foods and in their most natural form; eat three servings of vegetables daily, as well as at least three servings of fruit for dessert or snacks; eat at least five times a week beans

Table 1: Guidelines related to the amount of macronutrients and micronutrients of both groups participating in the research.

Food group	Amount of servings per day	Average caloric value per serving
Rice, bread, pasta, potato, and cassava	6	150 kcal
Vegetables	3	15 kcal
Fruits	3	70 kcal
Beans	1	55 kcal
Milk, cheese, and yogurt	3	120 kcal
Meat, fish, and eggs	1	190 kcal
Oil and fat	1	73 kcal
Sugar and candies	1	110 kcal

with rice because of their complete protein combination; consume three servings of milk and dairy products daily and a portion of meat, poultry, fish, or eggs, as well as removing the apparent fats from meat and poultry skin prior to preparation; ingest at most a portion of vegetable oil, olive oil, butter, or margarine; avoid soft drinks or industrialized juices, cakes, sweet and stuffed biscuits, desserts, and other goodies; decrease the amount of salt in the food and remove the salt shaker from the table; drink at least two liters of water daily at meal intervals. Practice thirty minutes daily of physical activity, avoiding alcohol and smoking. The macronutrient and micronutrient guidelines are contained in Table 1. Individuals in the S group included 14 g of *in natura* GB on meals. The choice of the amount of GB administered was based on a previous study conducted by Bucheli et al. [28] in volunteers between 65 and 70 years. In that study, Bucheli et al. showed positive effects in the use of GB. The authors concluded that dietary supplementation of goji berries for 30–90 days can prevent or reduce damage caused by free radicals by increasing antioxidant capacity in both 30 and 90 days, compared to the placebo group. Daily dietary supplementation with GB for 30–90 days increases plasma zeaxanthin and total antioxidant levels as well as protects from hypopigmentation and accumulation of soft drusen in the macula of elderly subjects [28]. Constituents of GB that have biological effect are zeaxanthin, rutin, betaine, cerebroside, β-sitosterol, flavonoids, amino acids, minerals, vitamins (in particular, riboflavin, thiamin, and ascorbic acid), and quercetin in addition to other phenolic compounds [21, 22]. The patients were passed through nutritional status and clinical, anthropometric, and biochemical evaluation at the beginning (baseline), 15 and 45 days after the supplementation had started.

2.3. Assessment of Anthropometric Measures. The anthropometric parameters, weight [29], height [29], and waist circumference [30, 31], were measured by a properly trained person, according to previously established criteria. The evaluation of the nutritional status was obtained by calculating the body mass index (BMI) [29]. The elderly ones had the nutritional status evaluated by BMI using the cutoff points defined for this age group [32].

2.4. Blood Sampling. Venous blood samples (20 ml) were obtained from participants after overnight fasting between 7:00 and 9:00 am. The samples were divided into three aliquots, in clot activator tube, EDTA (ethylenediaminetetraacetic acid), and sodium fluoride tubes in order to biochemical analyses and determination of redox state markers. Serum and plasma samples were obtained after centrifuging at 300g for 15 min at 4°C and were stored at −80°C until analyses.

2.5. Biochemical Assays. The serum concentrations of total cholesterol (TC) and triglycerides (TG) were determined by automated and colorimetric methods (Trinder's reaction; Labtest, Lagoa Santa, MG), and the HDL-C density was determined by homogeneous method (Labtest, Lagoa Santa, MG). LDL-C was estimated by the Friedewald equation (LDL-C = CT − (HDL-C + TG/5)) [33] or quantified by the homogeneous method (Labtest, MG) when the participant presented values of TG equal to or above 400 mg/dl. The LDL-C fraction was determined by the homogeneous LDL-C reagent (Labtest, Lagoa Santa, MG) after the selective precipitation of the other lipoproteins with heparin and magnesium, according to the procedure previously described by Hirano et al. [34] (VLDL), calculated by subtracting LDL and HDL from total cholesterol, and was determined by the values of serum cholesterol (Roche Diagnostics, Basel, BS, Switzerland). All measurements were carried out on the Cobas Mira Plus® automated equipment (Roche Diagnostics, Basel, BS, Switzerland). The concentrations of glucose (oxidase/peroxidase) were determined in automated equipment (Cobas Mira Plus, Roche Diagnostics, Basel, Switzerland) according to the manufacturer's specifications of the Labtest, Lagoa Santa, MG, kit. Serum concentrations of transaminases aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined by the continuous ultraviolet kinetic methods, easily adaptable to automatic analyzers of the Bioplus BIO 2000 brand. The methodology allows accurate and accurate results to be obtained. Quality control was performed with the PROEX biochemical controls of the National Quality Control Program (PNCQ), which is sponsored by the Brazilian Society of Clinical Analyzes (SBAC).

2.6. Oxidative Stress Biomarkers. Serum antioxidant capacity was determined according to the ferric reducing antioxidant potential (FRAP) assay as described by Benzie and Strain [35]. In this procedure, the antioxidants present in the serum were evaluated as reducers of Fe^{3+} to Fe^{2+}, which is chelated by 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) to form a Fe^{2+}–TPTZ complex with maximum absorbance at 593 nm. Ten microliters of serum were mixed with 1 mL of reagent containing 1.7 mM $FeCl_3$ and 0.8 mM TPTZ, prepared in 300 mM sodium acetate (pH 3.6). The samples were incubated for 15 min at 37°C, and the absorbance was read at 593 nm (Bioplus BIO 2000, Barueri, SP, Brazil). The 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was used as a standard, and the FRAP values were expressed as Trolox equivalents in micromoles per liter. Blood reduced

glutathione (GSH) was assessed according to the method proposed by Beutler et al. [36]. An aliquot of the total EDTA-treated blood was hemolyzed with cold water, and the proteins were precipitated by the addition of 30% trichloroacetic acid. Aliquots of 50 mL of the hemolyzed sample and 50 mL of 10 mM 5,50-dithiobis-2-nitrobenzoic acid were mixed in tubes containing 0.8 mL of 200 mM phosphate buffer, pH 8.0. After 3 min, the absorbance of the thiolate anion was measured at 412 nm (Bioplus BIO 2000, Barueri, SP, Brazil). GSH was used as a standard, and the results were expressed in micromoles per liter. Lipid peroxidation was performed by the detection of the oxidation product derivatives, substances that react with thiobarbituric acid, with malondialdehyde (MDA) being the most important, according to the previously described procedure. The absorbance of the supernatant was read at 532 nm [37]. The activity of the antioxidant enzyme catalase (CAT) and superoxide dismutase (SOD) was quantified in erythrocytes. Aliquots of the blood were centrifuged (700 g, 4°C, and 10 min), and erythrocytes were separated from the plasma. An equal volume of physiological solution was added, and the tubes were homogenized and centrifuged (700 g, 4°C, and 10 min). This procedure was repeated three times. Then, the erythrocytes were mixed with a hemolyzing solution (4 nM magnesium sulfate and 1 nM acetic acid), and the hemolyzed solution was stored for a maximum of 10 days at −80°C for analysis. CAT activity was quantified by monitoring the H_2O_2 decomposition in a spectrophotometer for 2 min at 240 nm [38]; its results were expressed in mmol/mg protein/second. SOD activity was assessed by the SOD-WST-SIGMA kit which allows the assay to be made using a water-soluble tetrazolium salt, WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2 (tetrazolium, monosodium salt), which produces a water-soluble formazan dye after reduction with a superoxide anion. The absorbance at 440 nm is proportional to the amount of the superoxide anion, the SOD activity was then measured by reducing the color at 440 nm, and the results were expressed as U/ml. Total proteins were determined according to a specific method, using bovine albumin as standard. Aliquots of the hemolyzed solution were added to the Bradford reagent and kept in the dark at room temperature for 5 min, and the absorbance reading was performed at 595 nm [39] with results expressed as $\mu g/\mu l$ protein.

2.7. Inflammatory Biomarkers. C-reactive protein (CRP) levels were determined by the nephelometry technique on BNII analyzer (Dade Behring, Marburg, Germany). The reagent consists of a suspension of polystyrene particles, coated with anti-PCR monoclonal antibody, which agglutinate in the presence of CRP from the sample. The intensity of diffused light in the nephelometer depends on the CRP concentration of the sample, so that, in comparison with dilutions of a standard of known concentrations, it is possible to determine the concentration of this mediator in the samples. The limit of the technique is 0.175 mg/l [40]. BD OptEIA diagnostic kits (Franklin Lakes, New Jersey, USA) were used for the determination of tumor necrosis factor (TNF-α), monocyte chemotactic protein 1 (MCP-1), interleukin 6 (IL-6), and interleukin-1β (IL-1β). All diagnostic kits consist of sandwich-type ELISA (immunoenzyme assay) and were performed according to the protocols provided by the manufacturer. Concentration calculations were performed using the equation of the straight line of each cytokine, obtained from calibration curves. The sensitivity of the assays is 2 pg/ml for TNF-α, 0.8 pg/ml for IL-1β, 2.2 pg/ml for IL-6, and 1.0 pg/ml for MCP-1.

2.8. Statistical Analysis. Continuous variables are presented as a mean and standard error of the mean, and categorical variables are presented as absolute frequency. All quantitative variables were tested for the normality by means of the Shapiro-Wilk test before analyses. Between groups, comparisons were analyzed by unidirectional ANOVA followed by the Bonferroni post hoc test; values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Participant Characteristics. Fifty participants were included (Table 1). Sociodemographic data demonstrated that, regarding gender, a similar distribution among groups was observed (72% women and 28% men in the C group and 68% women and 32% men in the S group). The average age in the C group was 49 years and in the S group 52 years. According to nutritional status, a division between adult and elderly individuals, in both groups, was performed; there were no patients with eutrophy, and the results of overweight and obesity are shown in Table 2. Regarding the use of the drugs, there were a similarity of consumption in both groups, especially the angiotensin-converting enzyme inhibitor (ACEI), angiotensin receptor blockers II (ARBs), and statins (Table 2).

3.2. Anthropometric Characteristics. When comparing the studied groups against the anthropometry and indicators of central obesity, significant differences were observed in relation to waist circumference (WC). The mean values were higher in group C when compared to those in group S; an interesting factor was that the supplemented group differed statistically at baseline against after a 45-day supplementation, reducing an average of 6.3 cm in the abdominal region during the 45-day study (Table 3).

3.3. Biochemical Assays. Regarding the results of the laboratory data, in relation to total cholesterol, a significant reduction was observed in group S between baseline and 45 days of supplementation, which does not occur in group C, characterized by oscillation of the values between the times analyzed (Figure 1(a)). For HDL-C, the statistical difference indicated improvement in the S group over the evaluated times, unlike the C group, in which there was oscillation during the study period. Similarly, the results for LDL-C and VLDL-C were statistically significant for group S between baseline and 45 days, indicating a reduction in this indicator, thus corroborating for a positive result in the four lipid variables: TC, LDL-C, HDL-C, and VLDL-C. In group C, an oscillation was observed for LDL-C and VLDL-C among the analyzed times, as shown in Figure 1. In relation to serum levels of TG (Figure 2(a)), both groups differed significantly

Table 2: Sociodemographic, anthropometric, and drug use characteristics in patients with metabolic syndrome, belonging to the control group (C) and supplemented group (S).

Variables	Groups	
	C ($n = 25$) Mean ± SE	S ($n = 25$) Mean ± SE
Age (years)	49.17 ± 2.56	52.6 ± 2.24
Age group*		
Up to 59 years	20 (80%)	19 (76%)
60 years and over	5 (20%)	6 (24%)
Gender*		
Female	18 (72%)	17 (68%)
Male	7 (28%)	8 (32%)
BMI (kg/m^2)	33.72 ± 1.28	32.98 ± 0.71
Adults*		
Eutrophy	—	—
Overweight	7 (33.3%)	5 (21.7%)
Obesity	14 (66.5%)	16 (73.8%)
Elderly*		
Eutrophy	—	—
Overweight	2 (50%)	2 (66.6%)
Obesity	2 (50%)	1 (33.3%)
Systolic blood pressure (mmHg)	128 ± 6	131.6 ± 3
Diastolic blood pressure (mmHg)	82 ± 3	84.8 ± 2
Drugs in use*		
ACEI/ARBs	20 (80%)	21 (84%)
Statins	13 (52%)	12 (48%)
Oral hypoglyclers	9 (36%)	7 (28%)
Diuretics	8 (32%)	7 (28%)

*Outcome presented in the form of n (%). SE: standard error; ACEI: angiotensin-converting enzyme inhibitor; ARBs: angiotensin receptor blockers II.

between baseline and 45 days, with a continuous and more significant reduction in group S. As for fasting blood sugar, the groups did not differ between themselves and between study times (Figure 2(b)). In this study, the plasma levels of AST and ALT were measured simultaneously, which presented similar trends. For AST and ALT values, groups C and S differed statistically between 45 days ($p < 0.05$ and $p < 0.01$, resp.). However, the improvement was observed in group S over the evaluated times (Figure 3).

3.4. Oxidative Stress Biomarkers. In relation to oxidative stress analysis, plasmatic lipid peroxidation was evaluated by the TBARS method (thiobarbituric acid reactive substances) and a reduction of 15% in group S between baseline and after 45 days of supplementation and an increase of 11.9% in group C was observed, considering the same experiment period (Figure 4(a)). Significant results for GSH were found for group S at the baseline and after 45 days of supplementation, presenting a 5.1% increase in values, in contrast to the results found in group C that remained without oscillation at the beginning and at the end of the experiment (Figure 4(b)). For total antioxidant, group S presented statistically significant results, indicating an increase of 175.3% between the baseline and after 45 days of supplementation, in contrast to group C, which maintained the results at the beginning and end of the experiment (baseline = 22.54 mM and after 45 days = 23.27 mM) (Figure 4(c)). The activity of the CAT enzyme showed statistically significant results for the S group, which presented a 22.6% increase between baseline and after 45 days of supplementation, unidentified evidence for group C, in which the results remained similar during the study period (Figure 4(d)). The results for SOD indicated a significant reduction of 39.1% in group S, comparing the baseline and after 45 days of supplementation. In contrast to the results found for group C where there was a reduction of only 4.1% among the evaluated times (Figure 4(e)).

3.5. Inflammatory Biomarkers. Regarding the analysis of the inflammatory profile in this study, although CRP presented a good inflammatory parameter, in this sample, there was no relationship between the groups and SM (Table 4). Results regarding the MCP-1 and TNF-α variables showed that the values did not oscillate between the times analyzed in both groups (Table 4). For the analyses of interleukins IL-1β and IL-6, the sensitivity of the method (0.8 pg/ml and 2.2 pg/ml, resp.) used to verify was not able to identify its presence in the samples.

4. Discussion

This study demonstrated that daily ingestion of 14 g of goji berry, a fruit rich in phenolic antioxidants [20–22], for 45 days increased the antioxidant protection of plasma and whole blood, thereby lowering oxidative stress in individuals with MS. Associated with improvement in the redox state, a significant reduction in abdominal fat was observed as evidenced by the reduction in waist circumference with an improvement in the lipid profile of the volunteers who received daily GB. It could be affirmed that the observed effects are due to the introduction in the diet of GB since both groups were composed of volunteers with MS and that they received the same orientation of diet based on recommendations according to the IV Brazilian Guidelines on Dyslipidemia and Atherosclerosis Prevention [27].

When analyzing the demographic data of the current study, it was observed that there is a predominance of females. Data from the National Cholesterol Education Program Adult Treatment Panel III (NCEP-ATP III) also show that 60% of women and 45% of North American men have a diagnosis of MS [41]. The results corroborate with other findings from Brazilian studies with a higher prevalence of MS in the female population [42–44].

The prevalence of overweight and obesity in all study participants support the relationship between subcutaneous adipose tissue and MS, and it is consistent with the literature. A research through computed tomography performed on 365 individuals, 187 patients with MS, and 187 patients without MS indicated that subcutaneous adipose tissue was significantly associated with MS in men and women, and it

Table 3: Anthropometric characteristics in patients with metabolic syndrome, belonging to the control group (C) and supplemented group (S).

Variables	Groups					
	C ($n = 25$) Mean ± SE Time of intervention (days)			S ($n = 25$) Mean ± SE Time of intervention (days)		
	Baseline	15	45	Baseline	15	45
Anthropometric characteristics						
Weight (kg)	91.59 ± 23.92	92.10 ± 24.11	92.43 ± 24.07	87.56 ± 15.42	87.24 ± 15.31	87.29 ± 15.34
BMI (kg/m^2)	33.72 ± 1.28	33.9 ± 1.29	34.03 ± 1.29	32.98 ± 0.71	32.87 ± 0.70	32.84 ± 0.70
WC (cm)	104.4 ± 16.92	104.14 ± 16.66	104.64 ± 17.34	106.46 ± 9.78	103.66 ± 9.69	100.08 ± 19.78***

Values are expressed as mean ± SEM (mean standard error). *** $p < 0.01$: significant difference between baseline and 45 days in the supplemented group.

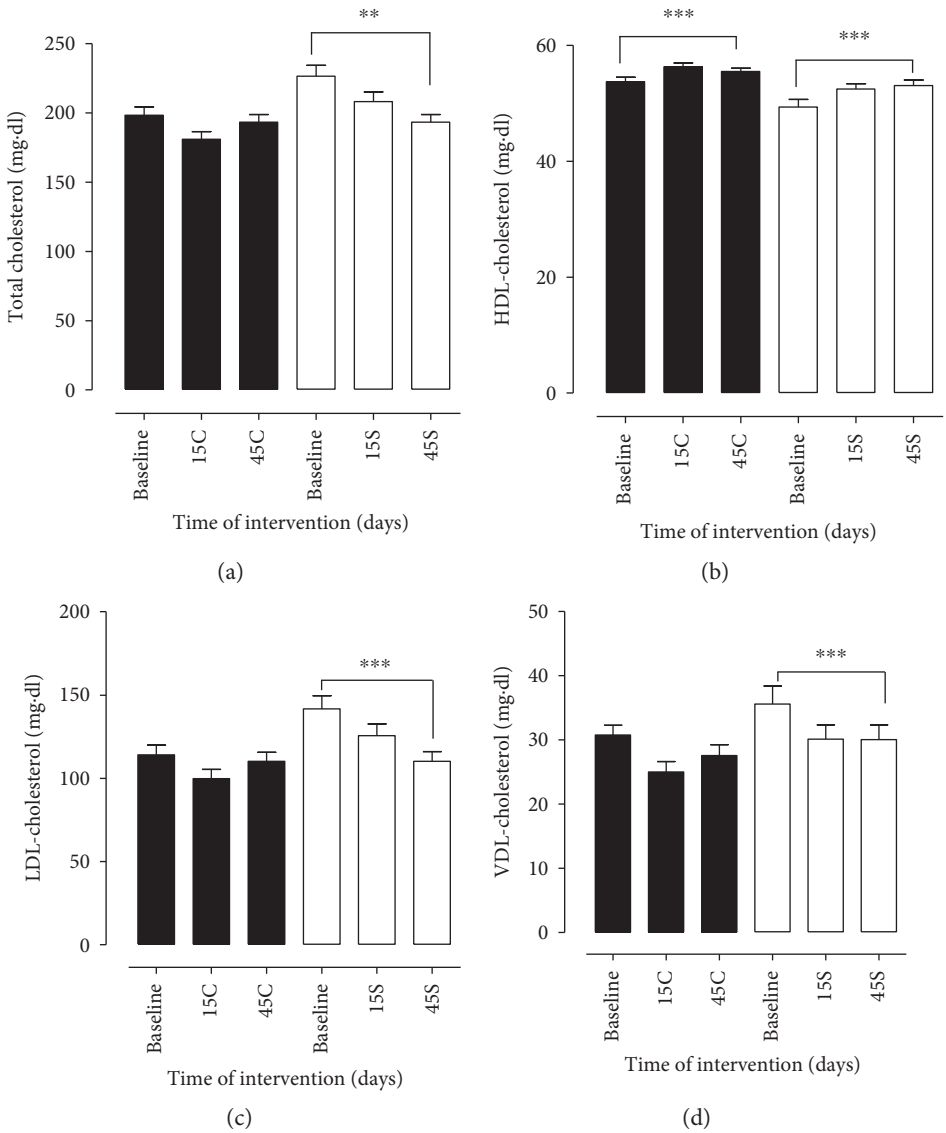

Figure 1: Lipid profile of patients with MS in the control group (C) and supplemented group (S) at the beginning, at day 15, and at day 45 after intervention. Total cholesterol (TC, (a)), HDL cholesterol (b), LDL cholesterol (c), and VLDL cholesterol (d) in patients with metabolic syndrome. Biochemical parameters were evaluated before (baseline) and 15 (15C and 15S) and 45 days (45C and 45S) after supplementation. Closed bars (control group—not supplemented) and open bars were supplemented with 14 g of goji berry daily. Values of ** $p < 0.01$ and *** $p < 0.001$ were considered statistically significant when comparing the baseline time with the end time of intervention in each group, using ANOVA followed by the Bonferroni post hoc test.

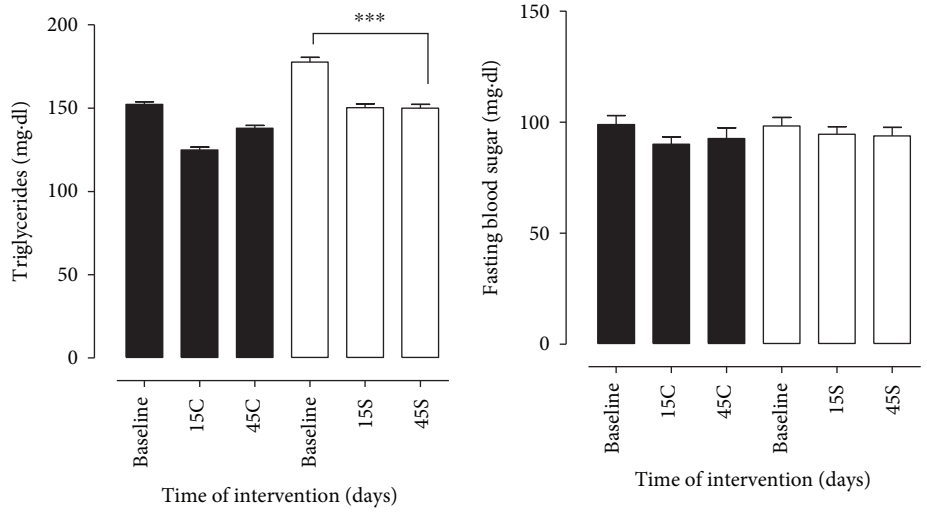

Figure 2: Serum triglycerides and fasting glycemia in patients with MS in the control group (C) and supplemented group (S) at the beginning, at day 15, and at day 45 after intervention. Evaluation of triglycerides (TG, (a)) and glucose (b) in patients with metabolic syndrome. Biochemical parameters were evaluated before (baseline) and 15 (15C and 15S) and 45 days (45C and 45S) after supplementation. Closed bars (control group—not supplemented) and open bars were supplemented with 14 g of goji berry daily. Values of $^{***}p < 0.001$ were considered statistically significant when comparing the baseline time with the end time of intervention in each group, using ANOVA followed by the Bonferroni post hoc test.

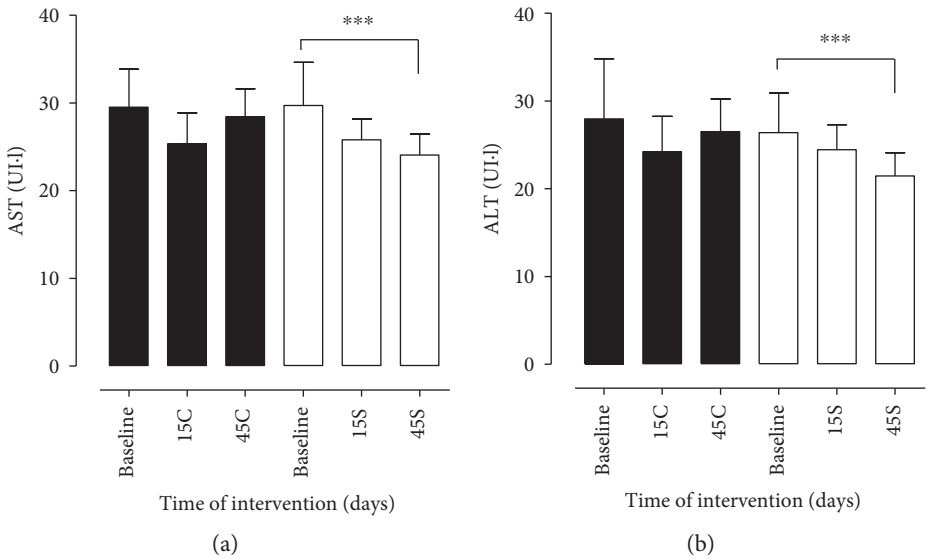

Figure 3: Hepatic enzyme patients with MS in the control group (C) and supplemented group (S) at the beginning, at day 15, and at day 45 after intervention. Evaluation of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in patients with metabolic syndrome. AST (a) and ALT (b) were evaluated before (group baseline) and 15 (15C and 15S) and 45 days (45S) after supplementation with 14 g of goji berry daily. The control group (closed bars) supplemented with goji (open bars). Values of $^{***}p < 0.001$ were considered statistically significant when comparing the baseline time with the end time of intervention in each group, using ANOVA followed by the Bonferroni post hoc test.

was also associated with inflammation increase and oxidative stress [45].

Corroborating the data cited in this study on waist circumference reduction, a study investigating the effects of quercetin that is found in onions (*Allium cepa* L.) on lipid profile and antioxidant status in moderately hypercholesterolemic individuals indicated that onion juice decreased significantly waist circumference, total cholesterol, and LDL-C. In addition, it increased the total antioxidant capacity, being recommended to combat cardiovascular diseases [46].

The data found in this study supports the hypothesis that the antioxidant effects of GB influenced the reduction of WC.

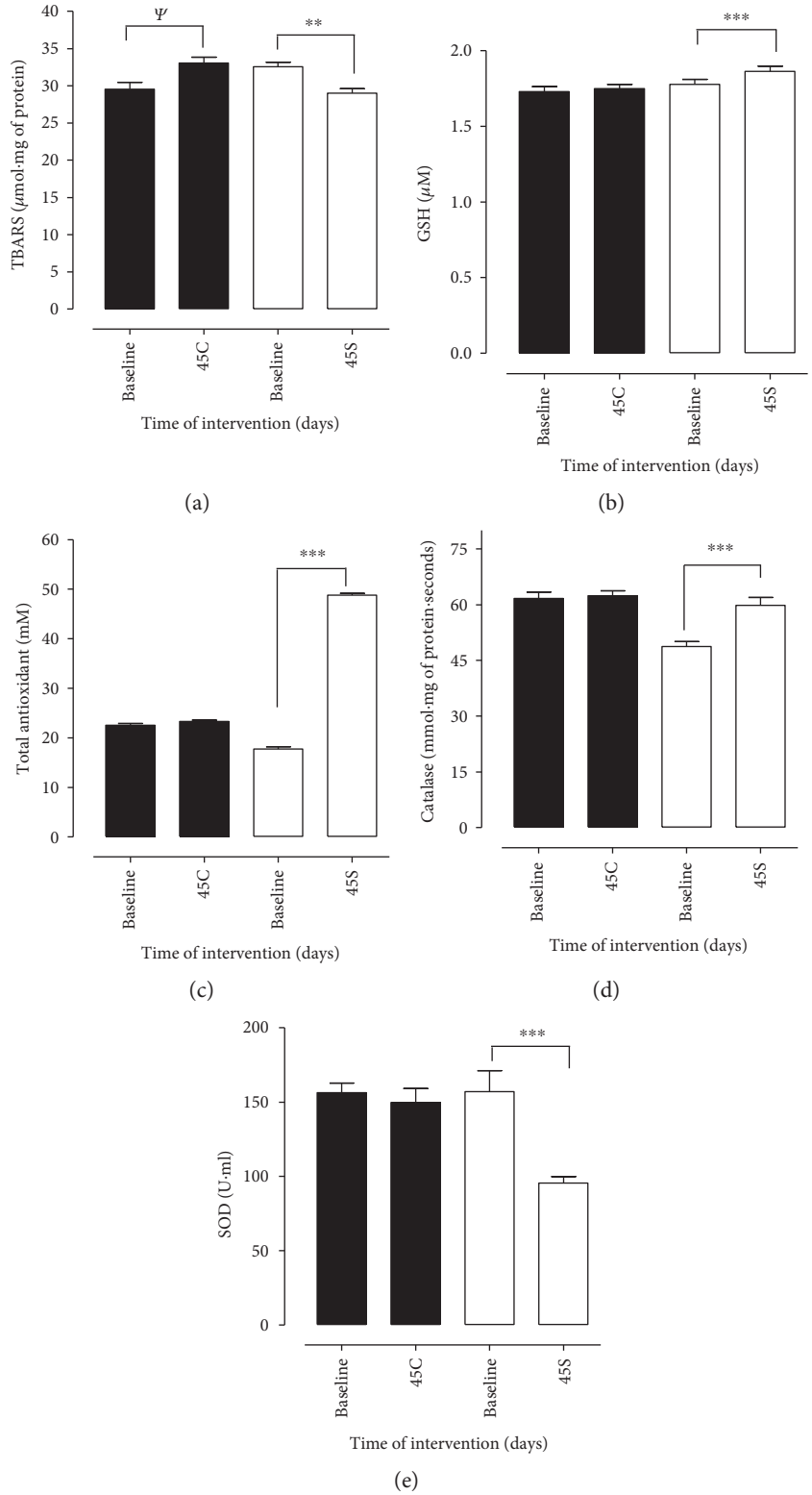

Figure 4: Oxidative stress variable patients with MS in the control group (C) and supplemented group (S) at the beginning and day 45 after intervention. Evaluation of thiobarbituric acid reactive substances (TBARS), blood reduced glutathione (GSH), total antioxidant control, catalase activity (CAT), and measurement of superoxide dismutase activity (SOD) in patients with metabolic syndrome. TBARS (a), GSH (b), total antioxidant control (c), and CzAT (d) were evaluated before (baseline) and 45 days after supplementation with 14 g of goji berry daily. Values of $^{**}p < 0.01$ and $^{***}p < 0.001$ were considered statistically significant when comparing the baseline time with the end time of intervention in each group, using ANOVA followed by the Bonferroni post hoc test.

Table 4: Inflammatory profile of patients with metabolic syndrome, belonging to the control (C) and supplemented (S) group, before and after the use of 14 g goji berry daily.

Variables	Groups			
	C ($n = 25$) Mean ± SE		S ($n = 25$) Mean ± SE	
	Time of intervention (days)			
Inflammatory profile	Baseline	45	Baseline	45
TNF-α (pg/ml)	38.88 ± 9.71	20.85 ± 4.41	18.98 ± 3.50	16.50 ± 1.47
MCP-1 (pg/ml)	21.09 ± 2.21	21.29 ± 3.16	16.44 ± 0.79	16.14 ± 1.00
CRP	4.66 ± 0.55	3.94 ± 0.41	3.72 ± 0.44	4.04 ± 0.48

Values are expressed as mean ± SEM (mean standard error).

The antioxidant action was also observed in a study of orange juice ingestion in different concentrations of polyphenols (299 and 745 mg/day) on the antioxidant defense system, oxidative stress, biomarkers, and clinical signs of MS in 100 individuals. A reduction in BMI, WC, and leptin was observed in the results, indicating the benefits of phenolic compounds [16] similar to those found in the present study with GB.

In relation to the lipid profile, in the supplemented groups with GB, the reduction of TG was sharper. Similar results were found in fish oil (3 g) research, rich in polyunsaturated fatty acids, indicating a reduction of TG in the group that used fish oil when compared to the control group (without substance use). In addition to this indicator, there was an increase in total antioxidant capacity [47], a fact that was also observed in our study with GB supplementation. The reduction in TG levels observed in the C group is probably due to the introduction in the diet of a greater quantity of fruits and vegetables since all the volunteers were directed to introduce a healthy diet as described in Materials and Methods.

The results presented in Figure 2(b) indicate that supplementation with GB was not able to improve the glycemic levels of the volunteers. However, it is observed that in both groups, the volunteers present glycemic values close to the reference values; this fact is probably due to the use of oral hypoglycemic agents since 100% of the volunteers are users of these drugs.

The findings of the current study indicate an improvement in serum levels or transaminases (ALT and AST) in the group supplemented with GB. Similar results were found in a study that investigated the effects of GB on alcohol-induced hepatic disease, and the authors found that the administration of this compound significantly inhibited the increase in serum AST and ALT activity caused by ethanol ingestion, and also, an increase in antioxidant protection (GSH, CAT, SOD, and GPx) and a reduction in lipid peroxidation were identified [48]. It could be inferred that the reduction in transaminase levels is associated with GB supplementation since group C shows no change in serum transaminase levels. Results like these demonstrate that the GB is a promising agent to protect the liver from hepatotoxicity and accumulation of liver fat.

In addition, evaluating hepatotoxicity, an experimental study with rats indicates that GB offers protection against acute hepatotoxicity caused by the drug paracetamol. Two groups were studied, one of which received a dose of 100 mg/kg of GB extract dissolved in physiological solution intraperitoneally for 7 days. After 7 days, a single dose of paracetamol (1 g/kg) was given to both groups. The serum markers of AST, ALT, total antioxidant capacity, and total oxidizing state were evaluated after 24 hours, and the group supplemented with GB presented better marker results [49].

The antioxidant/oxidative stress status in the evaluated groups (C and S) showed that in the S group, there was a significant reduction in the lipoperoxidation values evaluated by the plasma levels of TBARS (Figure 4(a)). The reduction of TBARS values in group S is probably associated with the consumption of GB, since associated with this a significant increase was observed in total antioxidant values in group S. Studies that evaluated the anti-inflammatory and antioxidant effects of GB, *Vaccinium marcrocarpon* (cranberry), and *Vaccinium myrtillus* (blueberry) extracts in rats showed a reduction in TBARS levels in all tested groups [21], a result that corroborates with the one found in this research with the supplementation of GB *in natura*.

Elevated levels of oxidative stress and adipokines are found in obese patients, associated with lower glutathione levels. In the present study, there was a significant increase in the GSH values in the S group after 45 days of supplementation, indicating an improvement in the oxidative profile. Similar results were found in another study, in which hepatic GSH values were significantly higher in animals treated with goji berry extracts than with cranberry [50].

The goji berry used in the study was able to significantly increase the total antioxidant activity in the supplemented group when compared to that in the control group (Figure 4(c)). The supplementation with antioxidants has the function of reducing oxidative stress because all nucleated cells respond to stress by positively regulating a complex set of defense mechanisms, a result observed in the present study through the daily insertion of GB into the diet.

Corroborating with the results of this study, a study conducted to evaluate the effects of vitamin C supplementation and antioxidant defenses presented data indicating that such supplementation causes a baseline increase in the expression of these defenses and modifies the responses of both muscle cells and lymphocytes to oxidative stress [51]. Similar results were found in the research using a combination of supplements with vitamins A, C, and E over a period of 8 weeks, having, as a result, the reduction of oxidative stress and indicating a potential mechanism underlying the protection of cardiovascular health and diabetes. Evidence from these

studies suggests that obesity directly influences the early development of insulin resistance and endothelial dysfunction, and it can be beneficially influenced by the ingestion of antioxidant foods [52].

Similar results were found for individuals with a high risk of developing cardiovascular diseases or dyslipidemias and human embryonic kidney-293 (HEK-293) cells. This effect were found after an increased consumption of foods rich in fiber, fruits, vegetables, or mate tea [14, 53, 54]. Our results corroborate with these studies since we observed an increase in the total antioxidant potential in volunteers who introduced 14 g of GB for 45 days (Figure 4(c)).

Studies have also shown that phenolic compounds from various sources are effective in improving antioxidant enzymes, suggesting their role in the prevention and treatment of oxidative stress-related diseases. Hence, the consumption of phenolic-rich fruits has been associated with reduced levels of ROS in animal experiments [55, 56]. Likewise, a high intake of phenolic-rich fruits has been reported to enhance the activities of the antioxidant enzymes [57].

Although studies have reported an increase in the activity of antioxidant enzymes in individuals who consume foods rich in phenolic compounds, our results show an increase in CAT activity and a significant reduction in SOD activity (Figures 4(d) and 4(e)). These findings may be related to the effect of quercetin, zeaxanthin, and rutin, the main phenolic constituents of GB [20–22] since studies report that the antioxidant effect of phenolic compounds could be associated with prevention in the formation of reactive species or in the improvement of enzyme activity antioxidants [58].

This decrease in SOD activity may be associated primarily with the antioxidant components of GB that may act directly preventing the formation of the superoxide anion radical since some studies show that antioxidant-rich fruit extracts are able to increase the expression of mRNA to CAT without changing the mRNA expression for SOD [10, 14]. In previous studies, we showed that the extract of GB presents high scavenging capacity against the DPPH radical; this shows its ability to act as a scavenger of reactive species [21], and this could explain the reduction of SOD activity in the supplemented group. In addition, the reduction of SOD activity may be explained by the ability of GB to increase other SOD isoforms such as Mn-SOD (SOD2) found in the mitochondrial matrix or Fe-SOD (SOD3) found in the extracellular medium [58]. In our study, we determined only the CuZn-SOD isoform (SOD1), which is found in erythrocytes.

In assessing the results of the inflammatory profile, despite CRP, MCP-1, and TNF-α presented as good inflammatory parameters, in this study, there was no relationship observed between the groups and MS during the evaluated period. For the analyses of IL-1β and IL-6, the sensitivity of the method used to verify was not able to identify its presence in the samples. These findings may be associated with the medications used by both groups (C and S) since all patients use oral hypoglycemic agents (metformin and glibenclamide) and statins. Studies show that metformin, glibenclamide, and statins reduce the production of the inflammatory cytokine IL-1β and CRP and the expression of TNF-α [59–61].

5. Conclusion

The results of this study demonstrated an increase in serum antioxidant capacity and GSH and a decrease in lipid peroxidation, LDL cholesterol, and waist circumference after long-term ingestion of GB, suggesting that this is an effective dietary supplement for the prevention of cardiovascular diseases in individuals with MS following a free diet.

Abbreviations

ACEI: Angiotensin-converting-enzyme inhibitor
ALT: Alanine aminotransferase
ARBs: Angiotensin receptor blockers II
AST: Aspartate aminotransferase
BMI: Body mass index
CAT: Catalase
CRP: C-reactive protein
CVD: Cardiovascular disease
DBP: Diastolic blood pressure
DM2: Diabetes mellitus type 2
DNA: Deoxyribonucleic acid
DPPH: 2,2-diphenyl-1-picrylhydrazyl
EDTA: Ethylenediaminetetraacetic acid
FRAP: Ferric reducing antioxidant potential
GB: Goji berry
GPx: Glutathione peroxidase
GSH: Reduced glutathione
HDL: High-density lipoprotein
IL-1β: Interleukin 1 beta
IL-6: Interleukin 6
IL-18: Interleukin 18
LDL: Low-density lipoprotein
MCP-1: Monocyte chemotactic protein
MDA: Malondialdehyde
MS: Metabolic syndrome
RNS: Reactive nitrogen species
ROS: Reactive oxygen species
SBP: Systolic blood pressure
SOD: Superoxide dismutase
TBARS: Thiobarbituric acid reactive substances
TC: Total cholesterol
TG: Triglycerides
TNF-α: Tumor necrosis factor alpha
VLDL: Very low-density lipoprotein
WC: Waist circumference.

Conflicts of Interest

The authors declare that there are no competing interests regarding the publication of this paper.

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Review Article

Caveolin-1: An Oxidative Stress-Related Target for Cancer Prevention

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Aberrant oxidative metabolism is one of the hallmarks of cancer. Reactive species overproduction could promote carcinogenesis via inducing genetic mutations and activating oncogenic pathways, and thus, antioxidant therapy was considered as an important strategy for cancer prevention and treatment. Caveolin-1 (Cav-1), a constituent protein of caveolae, has been shown to mediate tumorigenesis and progression through oxidative stress modulation recently. Reactive species could modulate the expression, degradation, posttranslational modifications, and membrane trafficking of Cav-1, while Cav-1-targeted treatments could scavenge the reactive species. More importantly, emerging evidences have indicated that multiple antioxidants could exert antitumor activities in cancer cells and protective activities in normal cells by modulating the Cav-1 pathway. Altogether, these findings indicate that Cav-1 may be a promising oxidative stress-related target for cancer antioxidant prevention. Elucidating the underlying interaction mechanisms between oxidative stress and Cav-1 is helpful for enhancing the preventive effects of antioxidants on cancer, for improving clinical outcomes of antioxidant-related therapeutics in cancer patients, and for developing Cav-1 targeted drugs. Herein, we summarize the available evidence of the roles of Cav-1 and oxidative stress in tumorigenesis and development and shed novel light on designing strategies for cancer prevention or treatment by utilizing the interaction mode between Cav-1 and oxidative stress.

1. Introduction

The cumulative production of reactive species, particularly reactive oxygen species (ROS) and reactive nitrogen species (RNS), through either endogenous or exogenous insults is termed oxidative stress [1]. Oxidative stress has been reported to be implicated in the etiology and progression of multiple human diseases, such as neurodegenerative disease, inflammatory disease, cardiovascular disease, allergies, immune system dysfunctions, diabetes, and aging as well as cancer [1]. Reactive species play an important role in cancer etiology and progression and are being progressively elucidated [2–4]. Physiological levels of reactive species are crucial for ensuring cell survival. However, overproduction of reactive species is detrimental to cells and could induce tumorigenesis by oxidizing specific intracellular moieties, resulting in genetic mutations and activations of ontogenetic pathways that stimulate proliferation and neoplastic transformation [2–5]. Cancer cells are usually submitted to higher levels of reactive species as a result of aberrations in oxidative metabolism (e.g., impaired mitochondrial oxidative phosphorylation [6] and increased aerobic glycolysis [7]) and signaling pathways (e.g., Ras [8] and AKT [9] activation), which further stimulate the malignant phenotypes of death evasion, angiogenesis, invasiveness, and metastasis [10]. Therefore, antioxidant therapies or dietary

supplementations of antioxidants have gained considerable interests in cancer prevention [11–15]. In addition, cancer patients widely take antioxidant supplements in order to lower oxidative damages of chemotherapy and radiation therapies to normal tissues [16–19]. It is estimated that approximately 45–80% of breast cancer patients use antioxidant supplements after diagnosis or during breast cancer treatment [17]. Common antioxidants used by cancer patients include glutathione (GSH), β-carotene, vitamin A (retinoic acid), vitamin C (ascorbic acid), vitamin E, and selenium [11, 13, 14].

Caveolae are 50 to 100 nm flask-shaped invaginations of the plasma membrane which have important roles in membrane trafficking (endocytosis and transcytosis), maintaining membrane lipid composition and cell signal transduction [20]. Cav-1, the major structural protein of caveolae, functions as scaffolding protein [21] regulating multiple physiological processes including caveola biogenesis, vesicular transport, cholesterol homeostasis, and signal transduction [22]. Recently, Cav-1 has been demonstrated to be closely involved in tumorigenesis and development, affecting the proliferation [23, 24], survival [25, 26], apoptosis [27, 28], migration [29, 30], invasion [24, 31], metastasis [32, 33], autophagy [34, 35], transformation [36], anoikis [37, 38], and chemotherapy resistance [39, 40] processes of cancer cells. Cav-1 is generally regarded as a tumor suppressor, and studies have implicated loss of Cav-1 in the pathogenesis and progression of multiple human cancers [41–44]. For example, loss of Cav-1 in cancer-associated fibroblasts (CAFs) could result in an activated tumor microenvironment, thereby driving early tumor recurrence, metastasis, and poor clinical outcomes in breast cancer [45]. In contrast, overexpression of Cav-1 in CAFs predicted a good outcome [45]. On the other hand, Cav-1 is also regarded as an oncoprotein in some kinds of malignancies. It was reported that the expression of Cav-1 was related to poor prognosis in lung pleomorphic carcinoma [46] and prostate carcinoma [47, 48]. In addition, Liu's group demonstrated that Cav-1 exhibited a stage-dependent, functional fluctuation during the progression of pancreatic cancer [49].

Recently, increasing evidences have showed that the oxidative stress processes in cancer cells were closely associated with Cav-1 [50–52]. Cav-1 is suggested to be a potential oxidative stress-related target during oxidative stress-induced cancer initiation and development. Reactive species overproduced in oxidative stress processes could modulate the expression [53], degradation [54], posttranslational modifications [55–57], and membrane trafficking [58] of Cav-1. Meanwhile, Cav-1 also has a feedback regulation effect on oxidative stress status in tumor microenvironment [50, 52]. For example, Cav-1 could attenuate hydrogen peroxide-induced oxidative damages to lung carcinoma cells [52]. More importantly, emerging evidences have indicated that multiple antioxidants could exert antitumor activities in cancer cells and protective activities in normal cells by modulating the Cav-1 pathway [59–61]. For example, treatment with antioxidants (such as quercetin, N-acetyl-cysteine, and metformin) was reported to reverse Cav-1 loss-induced phenotypes, such as mitochondrial dysfunction, oxidative stress, and aerobic glycolysis in CAFs [60]. Altogether, these findings indicate that Cav-1 may be a promising oxidative stress-related target for cancer antioxidant prevention. Considering the critical role of oxidative stress in mediating the therapeutic efficacies of radiotherapy and chemotherapy, understanding the action mechanisms of Cav-1 during oxidative stress processes is important for improving the clinical outcomes and for developing novel diagnostic tools and drugs for cancer antioxidant prevention. Herein, we summarize the current evidence of the roles of Cav-1 and oxidative stress, as well as their interaction in tumorigenesis and progression.

2. Structure, Localization, and Biofunctions of Cav-1

Originally named to describe the cave-like invaginations on the cell surface, caveolae are 50–100 nm membrane microdomains which represent a subcompartment of the plasma membrane and are enriched in glycosphingolipids, cholesterol, and lipid-anchored membrane proteins [22, 62]. Caveolins are caveolae-resident structural and scaffolding proteins, which are critical for the formation of caveolae and their interaction with signaling entities [22]. Each caveola contains approximately 100 to 200 caveolin molecules. There are three isoforms of caveolins (Cav-1, Cav-2, and Cav-3), while Cav-1 is the principal component of caveolae. Cav-1 (21–24 KDa) is an integral membrane protein with an unusual hairpin-like conformation (Figure 1(a)). The N- and C-terminal cytoplasmic tails of Cav-1 are separated by a hydrophobic segment that does not cross the membrane [63–65]. Both the C- and N-termini face the cytoplasm [66]. Cav-1 oligomerizes through amino acids 1–101. This oligomerization region contains the scaffolding domain (juxtamembrane 82–101 amino acids). Cav-1 has been identified primarily as two isoforms, Cav-1α and Cav-1β, which differs in their N-termini and derives from alternative translation initiation sites [67]. The presence of Cav-1 has been verified in most cell types by biochemical and morphological experiments, including epithelial cells, endothelial cells, fibroblasts, smooth muscle cells, and adipocytes [22, 68, 69]. Although Cav-1 is expressed ubiquitously, the levels of its expression vary among different tissues [22]. Cellular organelles where Cav-1 is enriched include the mitochondrion, the nucleus, the Golgi complex, and the endoplasmic reticulum [21, 22].

As shown in Figure 1(b), Cav-1 is involved in the regulation of multiple physiological processes, including caveola biogenesis, vesicular transport, cholesterol homeostasis, lipid transport, mitochondrial function, and signal transduction [22]. Cav-1 is a molecular hub. It could integrate transduction and negatively regulate a variety of signaling molecules, which include mitogen-activated protein kinases (MAPKs), epidermal growth factor receptor (EGFR), Neu, Ras family GTPases, transforming growth factor-β (TGF-β), G-protein-coupled receptors (GPCRs), Src family kinases, endothelial nitric oxide synthase (eNOS), adenylyl cyclases, protein kinase A (PKA), and AKT [22, 70–74]. The caveolin-signaling hypothesis proposes that the interactions between Cav-1 and these

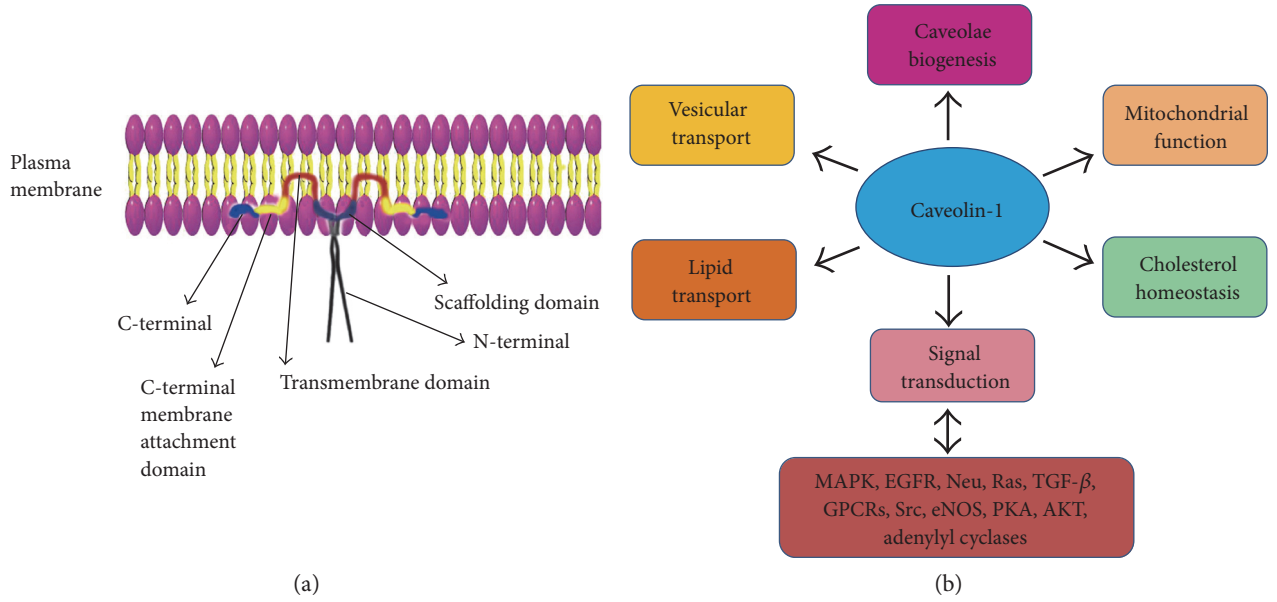


Figure 1: Structure and biofunctions of Cav-1. (a) The structure of Cav-1. The C-terminal, the C-terminal membrane attachment domain, and the transmembrane domain of Cav-1 are highlighted in blue, yellow, and red, respectively. The Cav-1-scaffolding domain (CSD) is responsible for Cav-1 interaction with other molecules. (b) Cav-1 biofunctions. Cav-1 is involved in the regulation of multiple physiological processes including caveola biogenesis, vesicular transport, cholesterol homeostasis, lipid transport, mitochondrial function, and signal transduction.

molecules are through the Cav-1-scaffolding domain (CSD) in Cav-1 molecule [72]. The Cav-1-scaffolding domain is also necessary for direct binding to cholesterol which participates in cholesterol transport and raft organization [75–77].

3. Emerging Role of Cav-1 in Cancer Prevention

As stated above, Cav-1 is a molecular hub-integrating transduction of multiple cellular molecules which are closely connected with the biological behaviors of cancer cells [78]. In addition, Cav-1 participates in multiple protein/protein and protein/lipid interactions which are critical for cell survival [22]. Undoubtedly, dysfunctional Cav-1 plays an important role in tumorigenesis and progression [79]. Human *CAV1* gene is localized to a suspected tumor suppressor locus (7q31.1) [53], which is a fragile genomic region and often deleted in cancers, suggesting that Cav-1 is possibly a tumor suppressor [80, 81]. The caveolin-signaling hypothesis proposes that Cav-1 could directly interact with multiple cancer-related signaling molecules including EGFR, Neu, TGF-β, Src, and AKT via the scaffolding domain and negatively modulate their aberrant activations. This hypothesis may further explain the role of Cav-1 as a tumor suppressor [22, 70]. Recent studies have implicated Cav-1 loss in the pathogenesis of various types of human malignancies [82–94] (Table 1). In breast cancer, loss of stromal Cav-1 was identified as a predictive biomarker of early tumor recurrence, lymph node metastasis, and tamoxifen-resistance as well as decreased survival in human breast cancer patients [42, 43, 95, 96], suggesting that Cav-1 functions as a tumor suppressor in breast cancer. Similar work confirmed that stromal Cav-1 loss was specifically associated with early ductal carcinoma in situ (DCIS) progression to invasive breast cancer, with shorter time to recurrence and higher recurrence rate [97]. In addition, Cav-1 is also negatively associated with breast cancer cells' transformation. Xie's group reported that Cav-1 expression was significantly attenuated in 3-phosphoinositide-dependent protein kinase-1- (PDK1-) mediated transformation of mammary epithelial cells [36]. Importantly, the prognostic value of stromal Cav-1 loss in breast cancers has now been independently validated [98–101] and has been extended to multiple types of human cancers, such as colorectal cancer [44], advanced prostate cancer [102], metastatic melanoma [41], gastric cancer [103], and osteosarcoma [104]. In colorectal cancer, it has been reported that stromal Cav-1 loss predicted poor survival [44]. In advanced prostate cancer, an absence of stromal Cav-1 was associated with metastatic disease and epithelial AKT activation [102]. In metastatic melanoma, stromal Cav-1 loss predicted poor survival of malignant melanoma patients [41]. In gastric cancer, epithelial Cav-1 loss could promote malignant progression of gastric cancer, and Cav-1 loss in CAFs heralded worse outcome of gastric cancer patient, suggesting Cav-1 level in CAFs may be a candidate therapeutic target and a useful prognostic marker of gastric cancer [103]. In osteosarcoma, Cav-1 downregulation could unleash c-Src and Met signaling, enabling osteosarcoma cells to invade neighboring tissues [104]. Conversely, overexpression of Cav-1 in osteosarcoma cell lines brought reduced malignancy with inhibited anchorage-independent growth, migration, and invasion. In addition, Cav-1 has also been reported to be closely connected with the multidrug resistance (MDR) of cancer cells [105–108]. For example, it was reported that Cav-1 could sensitize cancer cells to apoptosis in

Table 1: Caveolin-1 acts as a cancer suppressor in multiple malignancies.

Cancer type	Biofunction	Mechanisms	Reference number
Pancreatic cancer	Cancer suppressor	Inhibiting the EGFR, c-Raf, Mek, and Erk pathways; attenuating the expression of MMPs; inducing apoptosis; and inducing cell cycle arrest in the G0/G1 phase	[30, 90, 91]
Breast cancer	Cancer suppressor	Inducing apoptosis, inducing cell cycle arrest in the G2/M phase, suppressing glycolysis, downregulating the expression of growth factors, inhibiting lysosomal function, inhibiting autophagy, activating the *p53* pathway, inhibiting the AMPK pathway, and inhibiting the Ca^{2+}-activated potassium channels	[32, 34–36, 40, 42, 43, 95, 96, 98–101]
Colon cancer	Cancer suppressor	Inhibiting MMP-4 expression, facilitating cyclooxygenase-2, and EGFR degradation	[33, 44, 92, 93]
Melanoma	Cancer suppressor	Suppressing the integrin/Src/FAK pathway	[41, 94]
Leukemia cancer	Cancer suppressor	Inducing apoptosis, inducing the cell cycle arrest in the G1 phase, suppressing the PI3K/AKT/mTOR pathway, suppressing the VEGF redox signal transduction cascades	[82, 84]
Gastric Cancer	Cancer suppressor	Inducing the expression of E-cadherin, inhibiting fibroblast activation, suppressing the Ras/ MAPK pathway	[85–87, 103]
Rhabdomyosarcoma	Cancer suppressor	Suppressing the MAPK pathway, promoting muscular differentiation	[89]
Advanced prostate cancer	Cancer suppressor	Suppressing epithelial Akt activation	[102]
Osteosarcoma	Cancer suppressor	Inhibiting c-Src activity and the Met pathway	[104]

response to death stimuli, and a decrease of Cav-1 expression level was proved to contribute to chemotherapeutic cisplatin and carboplatin resistance [109].

Multiple clinical studies also verified the critical role of Cav-1 for cancer prevention. Bertino et al. observed that higher Cav-1 levels in tumor-associated stroma were significantly correlated with improved partial response rates ($P = 0.036$) and overall survival (OS) ($P = 0.008$) in advanced NSCLC patients, who treated with nanoparticle albumin-bound (nab) paclitaxel plus with carboplatin [110]. Furthermore, in a large cohort of 178 patients with colorectal cancer, Zhao et al. observed that the loss of stromal Cav-1 expression was associated with shorter disease-free survival ($P = 0.000$) and shorter OS ($P = 0.000$). This study further showed that the expression level of stromal Cav-1 was closely associated with histological type ($P = 0.022$), pathologic tumor-node-metastasis stage ($P = 0.047$), pathologic N stage ($P = 0.035$), and recurrence ($P = 0.000$) of colorectal cancer [44]. In a larger cohort of 724 patients with prostate cancer, Ayala and colleagues observed significantly decreased levels of stromal Cav-1 in concordance with increased Gleason score ($P = 0.012$) and reduced relapse-free survival ($P = 0.009$) [111]. Moreover, Jia et al. analyzed a total of 110 patients with esophageal squamous cell carcinoma and found that downregulation of stromal Cav-1 expression was associated with shorter disease-free survival ($P < 0.001$) and OS ($P < 0.001$), accompanied with more lymph node metastases ($P = 0.020$) and more local regional recurrences ($P = 0.002$) [112]. In addition, Yang et al. reported that increased Cav-1 expression was associated with prolonged overall survival rate in hepatocellular carcinoma ($P = 0.021$) [113]. Similar findings were also confirmed on breast cancer [99] and gastric cancer patients [114]. In particular, our analysis also revealed a close link between Cav-1 and lung cancer. Km-Plot analysis shows that lung cancer patients with Cav-1^{high} expression exhibit a better (OS) compared to those patients with Cav-1^{low} expression (Figure 2(a)). Meanwhile, high Cav-1 expression also indicates a better OS for lung adenocarcinoma but not for squamous cell lung carcinoma (Figures 2(b) and 2(c)). What is more, in both lung cancer patients with smoke history or no, Cav-1 exhibits a positive correlation with better OS, indicating that Cav-1-targeting strategy might be effective for lung cancer patients independent of the factor of smoke (Figures 2(d) and 2(e)). Interestingly, Cav-1^{high} expression also indicates an improved progression-free survival (PFS) and postprogression survival (PPS), further indicating the tumor suppressive role of Cav-1 in lung cancer (Figures 2(f) and 2(g)). In sum, accumulating evidence has indicated that Cav-1 might act as a tumor suppressor in multiple malignant tumors.

Interestingly, Cav-1 also acts as an oncoprotein depending on the tumor type and/or tumor stage. Particularly, Cav-1 seems to act as a tumor suppressor at early stages of cancer progression but as an oncoprotein in advanced-stage cancer [115]. For example, Cav-1 was found upregulated in multidrug-resistant colon cancer cells, adriamycin-resistant breast cancer cells, and taxol- and gemcitabine-resistant lung cancer cells [108, 116, 117]. Additionally, it was reported that *CAV1* silencing could sensitize breast cancer stem cells (CSCs) by limiting their self-renewal ability but promoting the differentiation process [118]. The conflict roles of Cav-1 in tumor progression may be partly explained by the observation that Cav-1 has several peptide domains with opposing functions [83]. Tyrosine phosphorylation, serine phosphorylation, and dominant-negative point mutations in these domains serve to functionally inactivate the tumor suppressor function of Cav-1 [83]. Meanwhile, we previously

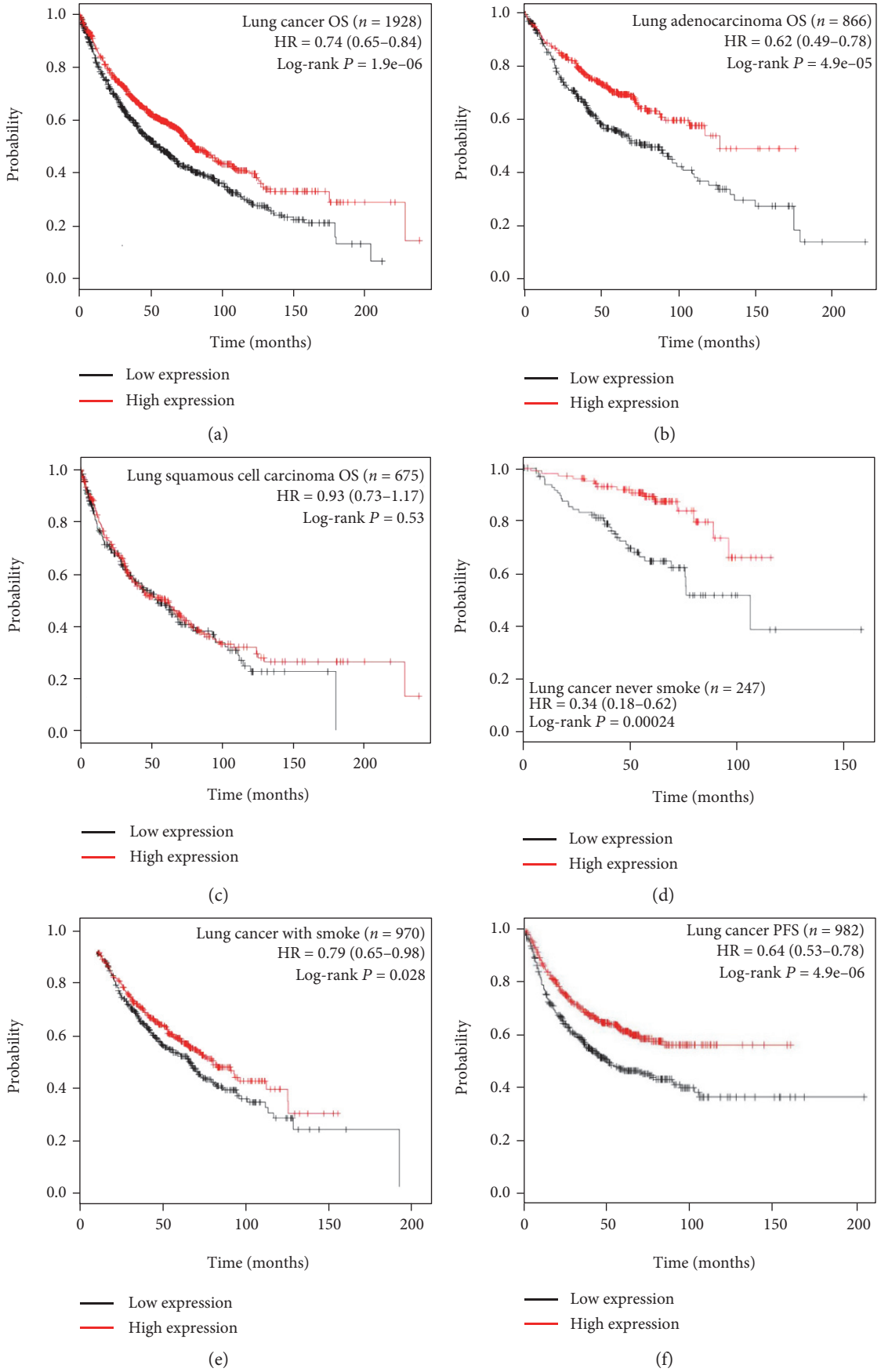
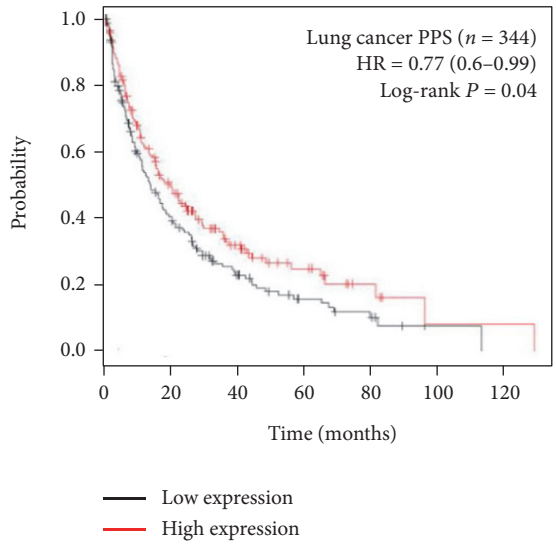


Figure 2: Continued.



(g)

Figure 2: Tumor suppressive role of Cav-1 in lung cancer by Km-Plot analysis. (a) Cav-1 predicts an improved OS among lung cancer patients; (b-c) Cav-1^{high} expression is closely correlated with improved OS in lung adenocarcinoma but not in squamous carcinoma; (d-e) the clinical significance of Cav-1 in predicting OS is independent of smoke history; (f-g) besides OS, Cav-1^{high} expression is also significantly correlated with improved PFS and PPS.

proposed a Cav-1 fluctuation model during cancer development [119]. It is suggested that Cav-1 might act as a kind of stress signal, which protects cells from hazardous damage, but its loss may make cells more sensitive to oncogenic events. However, when cancer progresses into the advanced stage or are treated with cytotoxic agents, the expression of Cav-1 would be upregulated to protect cancer cells escaping from death by speeding aerobic glycolysis, increasing stem cell population or overexpressing ABC transporters. Therefore, it is easy to understand current confusing evidence of Cav-1 in cancer development, and Cav-1 resurrection strategy is promising for preventing normal cells from malignant transformation.

4. Critical Role of Oxidative Stress Signaling in Mediating Cav-1 Anticarcinogenic Activities

Although physiological levels of reactive species function as important signaling of certain subcellular events such as enzymatic activity [5], gene expression [120], and protein synthesis [121], elevated levels of reactive species could initiate multiple toxic oxidative reactions including initiation of lipid peroxidation, direct inhibition of membrane sodium/potassium ATPase activity and mitochondrial respiratory chain enzymes, inactivation of glyceraldehyde-3-phosphate dehydrogenase, and membrane sodium channels [2–4]. All these toxicities are reported to play a role in carcinogenesis. In addition, elevated levels of reactive species could alter and damage many intracellular molecules, including nucleic acids, proteins, lipids, and polysaccharides [122], and therefore initiating a series of pathological processes and diseases. For example, reactive species could cause nicks in DNA, as well as malfunctions in the DNA repair mechanism. DNA oxidation induced by these reactive species generates 8-hydroxy-2′-deoxyguanosine, a product that is able to generate mutations in DNA and enhances carcinogenesis [123]. Furthermore, cancer cells usually exhibit increased levels of reactive species [124], which were found to facilitate cancer growth through sustained proliferation, apoptosis resistance, death evasion, angiogenesis, invasiveness, metastasis, and hypoxia-inducible factor 1 (HIF-1) activation [125–129]. Therefore, eliminating elevated oxidative stress is considered as an important strategy for cancer prevention.

Recent studies also revealed the critical role of oxidative stress in Cav-1 loss-mediated carcinogenic process. *CAV1* silencing in fibroblasts promoted ROS production, despaired mitochondrial activity, and DNA damage [50]. Similarly, treatment of fibroblasts with pro-oxidant buthionine sulfoxide caused the dose-dependent downregulation of Cav-1 [130]. In a coculture system, $CAV1^{-/-}$ fibroblasts were validated to promote breast cancer growth via driving aerobic glycolysis. Remarkably, the tumor-promoting effects of $CAV1^{-/-}$ fibroblasts were reverted by recombinant overexpression of superoxide dismutase 2 (SOD2), thus implying that oxidative stress is critical for facilitating Cav-1 loss-induced carcinogenesis [131]. How does Cav-1 loss promote oxidative stress and mitochondrial dysfunction? Previous studies have found that Cav-1 loss has a close link with the increased NO synthase (NOS) activity and NO production (Figure 3). Although physiological NO plays significant role in maintaining cellular normal functions and modulating inflammatory response, excessive NO production is deleterious, as it inhibits mitochondrial OXPHOS chain and induces the overproduction of superoxide anion, hydrogen peroxide, and peroxynitrite, which exert potent cytotoxic and carcinogenic effects. For example, peroxynitrite could lead to mutation in the *p53* tumor suppressor gene and initiate oncogenic events [132]. NO is synthesized by at least four

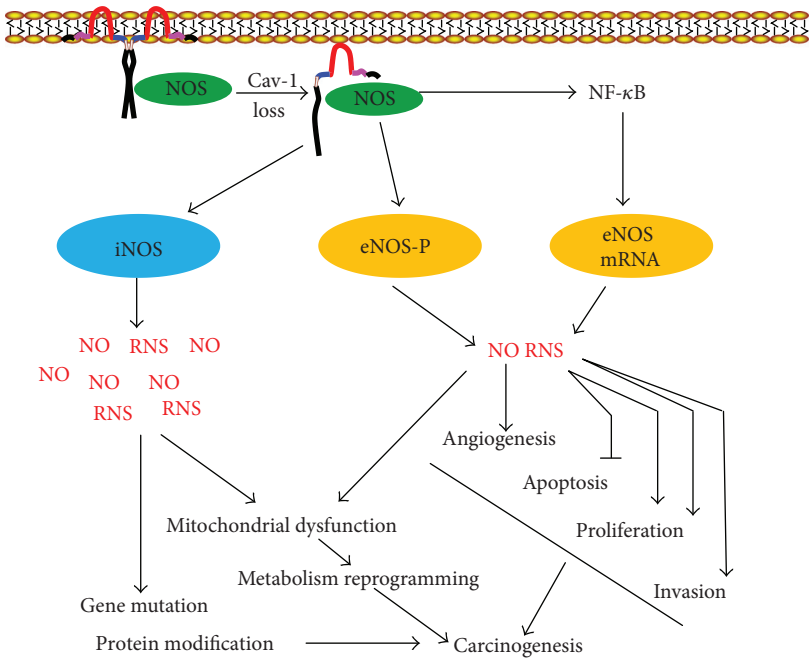


Figure 3: Cav-1 loss facilitates carcinogenesis through activating NOS activity. Cav-1 could directly interact with NOS enzymes through the scaffolding region. Following Cav-1 loss, eNOS will be released from the complex and activated through phosphorylation and mRNA overexpression, leading to the overproduction of NO and RNS. The high levels of NO and/or RNS would facilitate cell proliferation, apoptosis evasion, angiogenesis, and EMT process and finally induce carcinogenic transformation. Besides eNOS, iNOS will also be activated following Cav-1 loss. Since iNOS is capable of generating the micromolar level of NO, its activation will bring RNS burst and results in DNA damage and mitochondrial dysfunction, which finally promotes carcinogenesis.

isoforms of NOS enzyme: endothelial NOS (eNOS), inducible NOS (iNOS), neuronal NOS (nNOS), and, more recently, mitochondrial NOS. Mounting evidence has validated the increased NOS expression and/or activities, especially for the eNOS subtype, in human malignancies such as breast, central nervous system, and colon tumors [133]. eNOS was demonstrated to produce nanomolar level of NO, and its activation was validated to promote cancer angiogenesis cascade, apoptosis evasion, and epithelial-mensenchymal transition (EMT) process [134]. Interestingly, eNOS was demonstrated to directly interact with Cav-1 through its scaffolding domain [135]. Meanwhile, Cav-1 internalization has been shown to further regulate eNOS activation [136]. *CAV1*-deficient mice also displayed elevated eNOS expression and phosphorylation, and eNOS inhibitor could block Cav-1 loss-induced cancer growth, angiogenesis, and metastasis [137]. Since only iNOS produces micromolar NO concentrations, iNOS activation would bring much more influences on DNA damage and mitochondrial activity. Therefore, targeting iNOS for cancer prevention and treatment has also been extensively studied. iNOS expression was also revealed upregulated in multiple malignancies including colon, breast, prostate, bladder, and skin tumors [138]. Meanwhile, administration of iNOS selective inhibitor was also demonstrated to interrupt the development of AOM-induced aberrant crypt foci, accompanied by reduced expression of COX-2 and oxidative stress [139]. All these findings highlighted the significant role of NOS/NO signaling in mediating Cav-1 anticarcinogenic activities.

Besides NOS/NO signaling, genetic profiling assay also showed that silencing *CAV1* in stromal cells, 48 known ROS-related genes were upregulated, which are mainly associated with mitochondrial oxidative phosphorylation and peroxisome biogenesis. Furthermore, 45 HIF-target genes, 21 glycolytic enzymes, and 86 NF-κB-related genes were transcriptionally upregulated, implying that cellular metabolism was switched to glycolytic phenotype, which favors cancer initiation and growth [50]. ROS overproduction is closely associated with DNA damage response such as PARP. It was found that 5 PARP genes and 2 DNA-damage-induced transcripts Ddit3/Ddit4l were upregulated following CAV-1 knockdown [50]. Another genomic analysis also suggested that $CAV1^{-/-}$ stromal cells showed the upregulation of 55 genes associated with oxidative stress, accompanied with overexpression of 129 genes correlated with DNA damage/repair response [50]. All these results indicated that elevated ROS production following *CAV1* silencing might facilitate cancer formation through activating DNA damage response. It is interesting to elucidate the precise interaction mode between Cav-1 and oxidative stress.

5. Cav-1 and Oxidative Stress Regulation

Increasing studies have proved that Cav-1 is an oxidative stress-related protein, as reactive species could affect the expression, degradation, posttranslational modifications, and membrane trafficking of Cav-1 (Figure 4). Meanwhile, modulating the Cav-1 pathway could significantly affect the

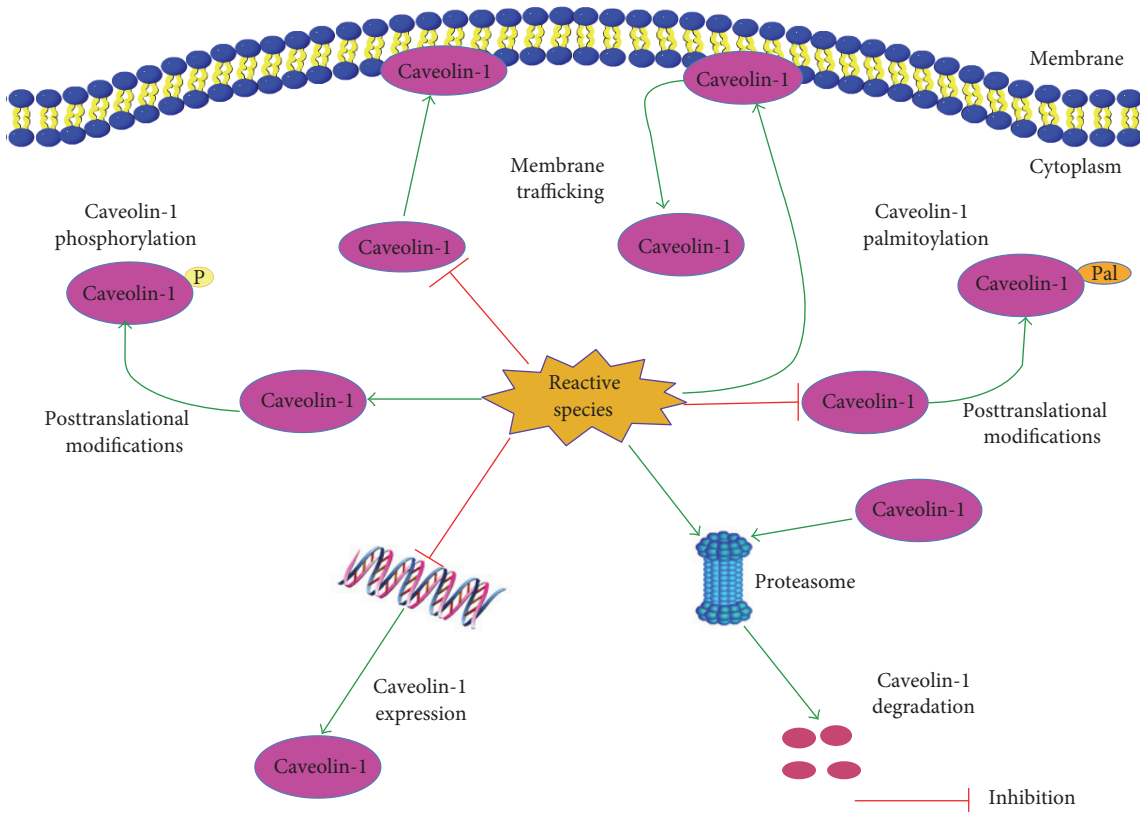


Figure 4: Cav-1 is an oxidative stress-related protein. On one hand, reactive species could decrease Cav-1 expression and induce its degradation through the proteasome pathway. On the other hand, reactive species could induce the phosphorylation but inhibit the palmitoylation of Cav-1. In addition, reactive species could induce Cav-1 internalization and inhibit the trafficking of newly synthesized Cav-1 to membrane raft domains.

oxidative stress status in normal and cancer cells. Therefore, Cav-1 may be a target of antioxidants in oxidative stress modulation and cancer antioxidant prevention. Herein, we summarize the available evidences about the implications of Cav-1 in the oxidative stress modulation effect of antioxidants and shed novel insights for Cav-1-targeted antioxidant therapy in cancer.

5.1. Cav-1 Is an Oxidative Stress-Related Protein. Intracellular reactive species are generated to serve as the second messengers, and some are linked to caveolae-signaling systems. Numerous cell surface receptors, which initiate a signal transduction cascade involving reactive species when activated by ligand binding, are recruited in caveolae [69]. These membrane microdomains, which play a pivotal role in signal transduction [140], therefore have been proposed to be a preferred site of reactive species generation [141].

5.1.1. Oxidative Stress Modulates the Expression and Degradation of Cav-1. Cav-1 reduction could significantly perturb the function of caveolae. In spite of Cav-1 reduction, caveolae are still able to be assembled at the plasma membrane, but their functions will be significantly impaired [142]. Oxidative stress could lead to the reduction of Cav-1 by modulating its expression and degradation (Figure 4). For example, Cai et al. reported that inhibitors of ROS could increase the expression of Cav-1 in human brain tumor microvascular endothelial cells [143]. Zhang et al. reported that high concentrations of glucose could decrease Cav-1 expression in lens epithelial cells [144]. Furthermore, Mougeolle and colleagues reported that hydrogen peroxide at nontoxic concentrations could increase the concentrations of reactive species in myoblasts and decrease the expression level of Cav-1. However, this phenomenon was not observed in the presence of a proteasome inhibitor, suggesting that Cav-1 was rapidly degraded by the proteasome [142]. Moreover, Luanpitpong and colleagues have found that superoxide anion and hydrogen peroxide could attenuate the expression of Cav-1 in lung carcinoma H460 cells [145]. Further mechanism studies indicated that the downregulation effect of superoxide anion and hydrogen peroxide on Cav-1 is modulated through a protein degradation mechanism via the ubiquitin-proteasome pathway [145].

5.1.2. Oxidative Stress and Posttranslational Modifications of Cav-1. Cav-1 is mainly subject to two kinds of posttranslational modifications that regulate its activity, including phosphorylation and palmitoylation. Phosphorylation of Cav-1 is closely connected with cell apoptosis and cell attachment during oxidative stress. Increased expression of p-Cav-1 is

antiapoptotic and may promote cell survival after oxidative stress [146, 147]. Cav-1 was first identified as a phosphoprotein in Rous sarcoma virus-transformed chicken embryo fibroblasts, which led to the hypothesis that Cav-1 may be a critical target during cellular transformation [148]. Since then, Cav-1 has been commonly identified as a phosphoprotein [56, 149–151]. Normally, Cav-1 is phosphorylated at a low [149, 150] or undetectable level [56, 151] in unstimulated cells. Reactive species generation could promote Cav-1 phosphorylation (Figure 4) [152]. For example, Volonte et al. suggested that multiple cellular stresses including high osmolarity, hydrogen peroxide, and UV light could induce the tyrosine 14-phosphorylation of Cav-1 [56]. Sun's group reported that ROS overproduction induced by high glucose-containing medium could time dependently increase Cav-1 phosphorylation in podocytes [153]. In addition, it was reported that an increased tyrosine-14 phosphorylation of Cav-1 was detected in human umbilical vein endothelial cells (HUVECs) after treatment with 100 μM hydrogen peroxide for 30 minutes [154]. This increased tyrosine phosphorylation could be inhibited by tyrosine kinase inhibitors (herbimycin or genistein), which is consistent with the finding of Volonte [56]. What is more, Cav-1 could also be phosphorylated on serine-80 [155], and recent evidences suggested that serine phosphorylation of Cav-1 might be inversely correlated to tyrosine 14-phosphorylation [156].

Palmitoylation is the regulated, posttranslational modification of cysteine, serine, or threonine residues by the saturated fatty acid palmitate (e.g., palmitic acid), through a covalent thioester linkage [157]. Palmitoylation enhances the hydrophobicity of proteins and contributes to their membrane association. Palmitoylation typically occurs in membrane proteins and plays an important role in subcellular trafficking of proteins between membrane compartments, as well as in modulating protein-protein interactions [158]. Like many caveolae-targeted proteins, Cav-1 could also be acylated. Three cysteine residues near the C-terminus of Cav-1 (cysteines 133, 143, and 156) are susceptible to palmitoylation [159]. Cav-1 palmitoylation is required for cholesterol binding and transport to caveolae and for interaction with c-Src [160, 161]. Mutation of the cysteine residues could impair Cav-1 interaction with other acylated proteins [161] and its binding and transport of cholesterol. It has been reported that reactive species, such as hydrogen peroxide, could markedly inhibit the palmitoylation of Cav-1 (Figure 4) [150]. Moreover, the effect of hydrogen peroxide on Cav-1 palmitoylation usually occurs at much lower concentrations than those required to affect Cav-1 phosphorylation, indicating that the effect on palmitoylation is not a direct consequence of the increased phosphorylation.

5.1.3. Oxidative Stress and the Membrane Trafficking of Cav-1. The function and activity of Cav-1 are tightly regulated by posttranscriptional modification as well as its subcellular localization. An early recognition that Cav-1 may be a sensitive target of oxidative stress modulation originated from the observation that oxidation of caveolar cholesterol by cholesterol oxidase could cause a reversible translocation of Cav-1 from the caveolae to the Golgi apparatus [162]. By applying immunofluorescence assay, Kang et al. have demonstrated that an exposure to high doses (1 mM) of hydrogen peroxide could induce Cav-1 internalization in NIH3T3 fibroblasts [58]. Similarly, by carrying out biochemical experiments, Blair's group also found that treatment of endothelial cells with oxidized LDL, but not native LDL, could lead to the translocation of Cav-1 from plasma membrane caveolae to an intracellular membrane fraction [163]. In addition, metabolic labeling experiments also showed that hydrogen peroxide could inhibit the trafficking of newly synthesized Cav-1 to membrane raft domains in endothelial cells [150], and impairment of Cav-1 synthesis by hydrogen peroxide was not responsible for the diminished trafficking. Altogether, these results indicate that oxidative stress could affect the membrane trafficking of Cav-1 (Figure 4).

5.2. The Regulation Effects of Cav-1 on Oxidative Stress. Cav-1 also has a feedback regulation effect on oxidative stress status in cells (Figure 5). Shiroto and colleagues found that *CAV1* silencing in endothelial cells could increase the production of ROS in mitochondria and induce oxidative stress [164]. 2-deoxy-D-glucose, a glycolytic inhibitor, attenuated this increase, suggesting Cav-1 is in control of oxidative stress through glycolytic modulations [164]. Pavlides and colleagues have demonstrated that Cav-1 loss could induce oxidative stress, mimic hypoxia, and drive inflammation in the tumor microenvironment [50]. Martinez-Outschoorn et al. found that Cav-1 loss could lead to mitochondrial dysfunction, oxidative stress, and aerobic glycolysis in cancer-associated fibroblasts [60]. Wang et al. reported that Cav-1 knockdown resulted in eNOS redistribution to the perinuclear region and nearly doubled insulin-induced NO production in vascular endothelial cells [165]. Since NO is a competitive inhibitor of oxygen in the cytochrome oxidase present in mitochondrial complex IV, increased NO production could result in the inhibition of mitochondrion by attenuating the terminal phase of the mitochondrial electron transport chain complex, leading to electron leakage, superoxide formation, and mitochondrial dysfunction (Figure 5).

Cav-1 is highly expressed in endothelium. Therefore, the enriched Cav-1 in tumor vessels provides an interesting opportunity for Cav-1-targeted therapies. Gratton et al. demonstrated that a cell-permeable peptide derived from the CSD of Cav-1 could regulate microvascular permeability via inhibiting eNOS, and consequently markedly reduced tumor progression in mice [166]. Similarly, Suchaoin et al. reported that lung cancer H460 cells stably transfected with *CAV1*-overexpressing plasmids (H460/Cav-1) exhibited decreased ROS signal, while *CAV1*-specific shRNA-transfected (H460/shCav-1) cells showed enhanced ROS signal [52]. Interestingly, Pongjit et al. also established stable Cav-1-overexpressing (H460/Cav-1) cells and investigated the role of Cav-1 in modulating the oxidative stress induced by cisplatin. They found that overexpression of Cav-1 generated significantly higher superoxide anion level and could sensitize cisplatin-induced lung carcinoma cell apoptosis. Meanwhile, lung cancer H460 cells transfected with *CAV1*-specific shRNAs exhibited decreased

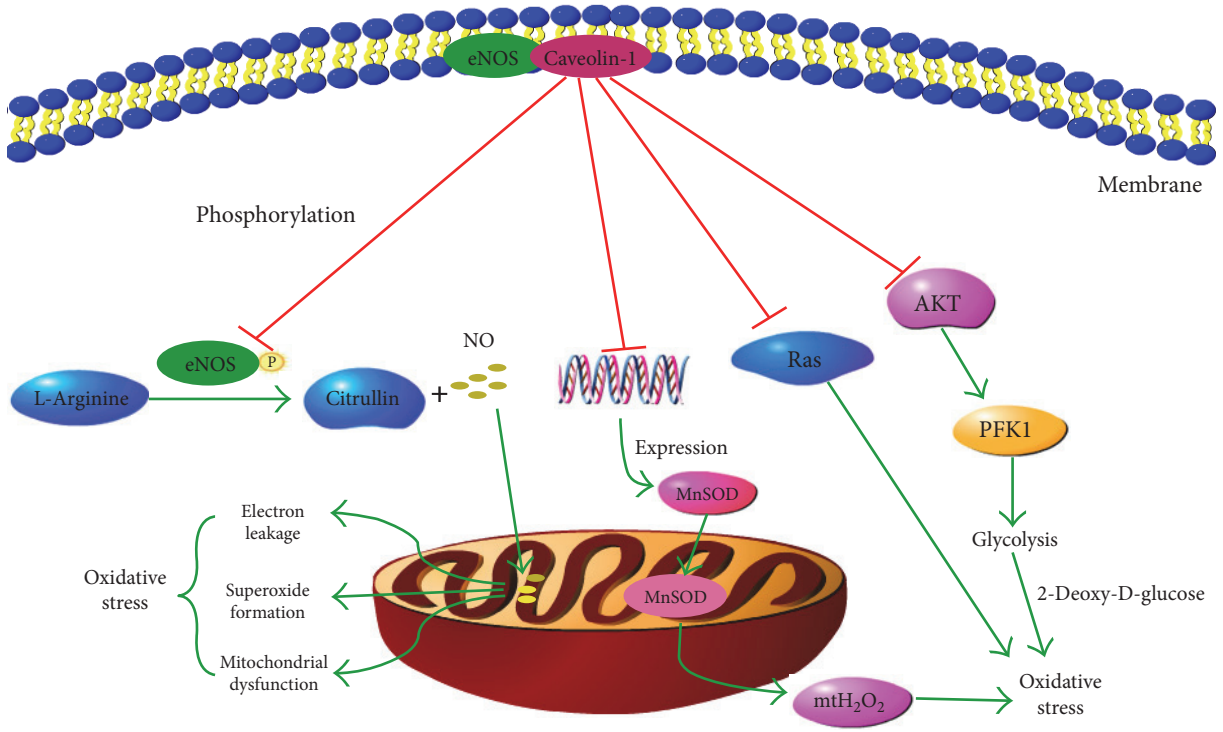


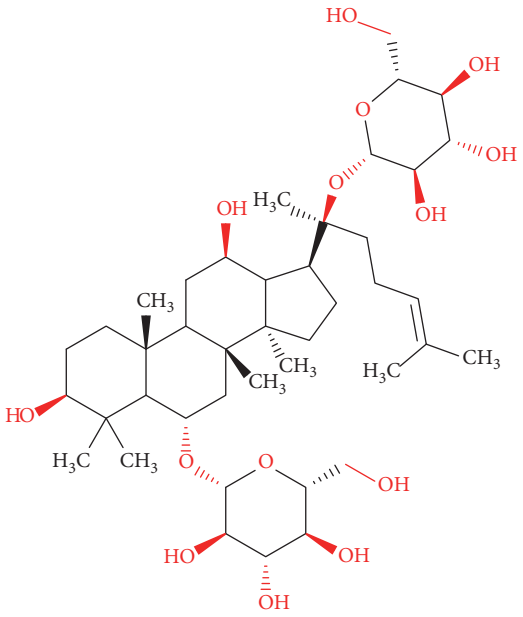
Figure 5: Regulation mechanisms of Cav-1 on oxidative stress. eNOS is maintained in an inactive state in caveolae by its interaction with Cav-1-scaffolding domain. Upon stimulation, eNOS is released and phosphorylated and could catalyze the NO synthesis reaction. NO overproduction could result in the inhibition of mitochondrion by attenuating the terminal phase of the electron transport chain complex, leading to electron leakage, superoxide formation, and mitochondrial dysfunction. Furthermore, Cav-1 could inhibit the transcription of MnSOD and thus reduce the release of excess mitochondria-derived H_2O_2 (mtH_2O_2). Moreover, glycolysis process could lead to the overproduction of reactive species, while 2-deoxy-D-glucose, a glycolysis inhibitor, could prevent reactive species burst. Phosphofructokinase 1 (PFK1) is the rate-limiting enzyme of the glycolysis process. Cav-1 could inactivate the PI3K/AKT/PFK1 pathway and therefore block the glycolysis process. Finally, Cav-1 could prevent the overproduction of reactive species by inactivating the Ras pathway.

superoxide generation and decreased cisplatin susceptibility [27]. Although these results revealed a heterogeneous outcome of *CAV1* overexpression/silencing to oxidative stress modulation, they revealed the feedback regulation pattern between Cav-1 and oxidative stress.

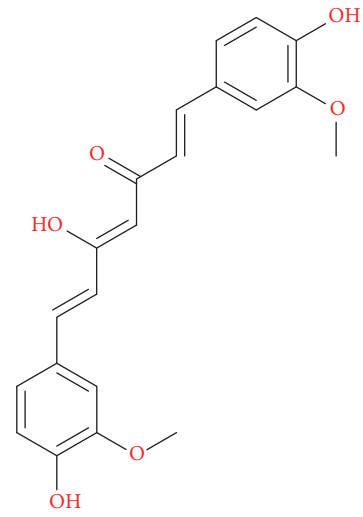
5.3. Modulation Effects of Antioxidants on Cav-1. As described above, reactive species could modulate the expression, degradation, posttranslational modifications, and membrane trafficking of Cav-1, while Cav-1-targeted treatments could scavenge the reactive species. More importantly, emerging evidence has indicated that multiple antioxidants could exert antitumor activities in cancer cells as well as protective activities in normal cells by modulating the Cav-1 pathway (Figure 6).

In terms of cancer cells, Kitano et al. reported that vitamin K3 analogs could induce selective tumor cytotoxicity in neuroblastoma by inducing Cav-1 expression [59]. Yang et al. proved that resveratrol could induce cytotoxicity in a hepatocellular carcinoma model by increasing the expression of endogenous Cav-1 [167]. Salani et al. found that metformin exerted antiproliferative effect in NSCLC cells by inducing the expression of Cav-1 [61]. Martinez-Outschoorn and colleagues reported that treatment with antioxidants (such as N-acetyl-cysteine, metformin, and quercetin) or NO inhibitors was sufficient to reverse many of the Cav-1 loss-induced phenotypes, such as mitochondrial dysfunction, oxidative stress, and aerobic glycolysis in cancer-associated fibroblasts [60].

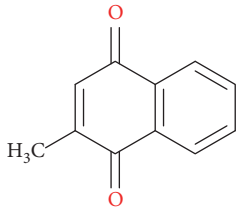
In terms of normal cells, Zhan et al. reported that ginsenoside Rg1 exerted protective effect on bleomycin-induced pulmonary fibrosis in rats by increasing the expression of *CAV1* mRNA and protein [168]. Nakaso et al. reported that knockdown of Cav-1 and/or Cav-2 prevented the protective effects of tocotrienol in a cellular Parkinson's disease model [169]. In addition, antioxidants such as lycopene [170] and quercetin [171] were also able to induce the expression of Cav-1. In particular, the effects of curcumin on Cav-1 pathway have been deeply reported. For example, Zeng et al. reported that curcumin exerted the antiproliferative effect in airway smooth muscle cells by upregulating the expression of Cav-1, while the disruption of caveolae using methyl-β-cyclodextrin attenuated the antiproliferative effects of curcumin [172]. Sun et al. reported that curcumin could prevent EMT of podocytes by suppressing the phosphorylation of Cav-1 and increasing stabilization of Cav-1 and β-catenin [173]. In addition, curcumin could inhibit renal inflammatory gene expression in vivo by reducing Cav-1 phosphorylation at Tyr (14) [174]. Yuan et al. investigated the mechanism of curcumin in increasing Cav-1 expression and found that



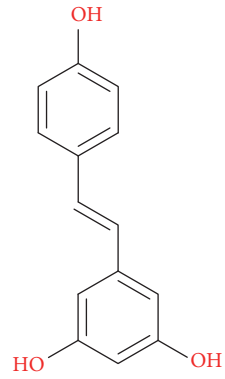
Ginsenoside Rg1



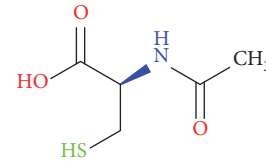
Curcumin



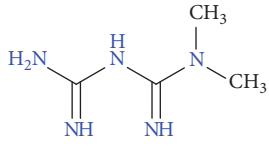
Vitamin K3



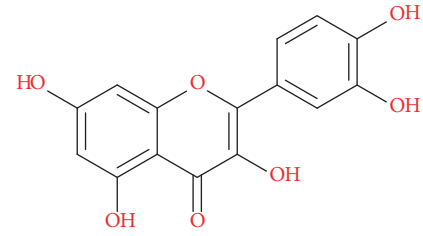
Resveratrol



N-Acetyl-cysteine



Metformin



Quercetin

Figure 6: Chemical structures of antioxidants influencing Cav-1 expression.

curcumin increased the expression of Cav-1 by inhibiting nuclear translocation of SREBP-1 [175]. In addition, curcumin could suppress ROS overproduction, which was believed to participate in blocking high-glucose-induced apoptosis of podocyte through regulating Cav-1 phosphorylation in both in vitro and in vivo experiments [153]. Altogether, the above evidences suggest that Cav-1 is a target of antioxidants in oxidative stress modulation, further proving that Cav-1 is an oxidative stress-related target for cancer antioxidant prevention.

Although antioxidant treatments have been explored for decades as attractive cancer prevention strategies, no antioxidant treatment regimen has reached the clinic at present. Notably, some preclinical and clinical trials even suggested that the cancer prevention potential of antioxidants was contrary to the expected outcome [176]. For example, Sayin and

colleagues recently reported that supplementing the diet with antioxidants N-acetylcysteine and vitamin E markedly increased tumor progression and reduced survival in mouse models of lung cancer [177]. The very large "Selenium and Vitamin E Cancer Prevention Trial" found no initial reduction in the risk of prostate cancer in healthy individuals taking either selenium or vitamin E supplements [178]. Indeed, long-term follow-up studies of these individuals suggested that vitamin E supplementation significantly increased the risk of prostate cancer among healthy men [179]. The disappointing results might be attributed to the multiple targets of antioxidants. Meanwhile, the heterogeneous in the dose, type, schedule, and action mechanisms of different antioxidant drugs might also have great influence on the endpoint. What is more, the poor solubility, stability, and limited bioavailability of some antioxidants also significantly impact on their therapeutic efficacy. Therefore, development of Cav-1-specific targeting antioxidant might be a novel strategy to improve the antioxidant prevention effects.

6. Therapeutic Implication of Cav-1-Targeted Treatment for Cancer Antioxidant Prevention

For many years, researchers have theorized that cancer cells depend on the activation of oncogenes or the inactivation of tumor suppressor genes for their survival. This theory is known as "oncogene addiction" [180]. However, recent studies have shed light on the vital mechanisms that ensure the survival of cancer cells, including the ability to escape from immune surveillance as well as the ability to undergo metabolic adaptations that provide cancer cells with a secure energy supply for their uncontrolled proliferation. Thus, targeting the "cart" (e.g., metabolism) rather than the "horse" (oncogenes and tumor suppressor genes) may be a more promising strategy for eliminating cancer cells while sparing normal cells [181]. Cancer cells undergo a metabolism switch, characterized by impaired mitochondrial oxidative phosphorylation [6] and increased aerobic glycolysis [7]. The metabolic reprogramming of malignant cells may be a "cart", and targeting the aberrant metabolism has been perceived as a more promising strategy for cancer prevention and treatment. As previously stated, aerobic glycolysis could lead to the overproduction of carcinogenic reactive species [7, 182], while Cav-1 could regulate cancer cell metabolism via suppressing MnSOD-driven glycolysis [183]. Meanwhile, Gene microarray analysis revealed that $CAV1^{-/-}$ stromal cells showed the upregulation of 48 genes associated with ROS production, 45 genes regulated by HIF-1, and 21 genes involved in glycolysis pathway [184]. In addition, $CAV1^{-/-}$ stromal cells exhibited defective mitochondrial functions due to the overexpression of NO [185]. Coculture of $CAV1^{-/-}$ stromal cells with cancer cells significantly promoted tumor growth and angiogenesis, while glycolysis inhibitor treatment greatly blocked the positive effects of $CAV1^{-/-}$ stromal cells [50, 185]. All these results indicated that Cav-1 may be a critical molecule in linking mitochondrial dysfunction, reactive species elevation, glycolysis enhancement, and finally carcinogenesis promotion. Therefore, therapeutic strategies restoring Cav-1 expression in both cancer cells and adjacent stromal cells should block cancer development via inhibiting glycolytic activity and reactive species overproduction.

On the other hand, compared with normal cells, cancer cells exhibit elevated levels of reactive species. Excessive reactive species not only induce oxidative damages and inactivation of tumor suppressor genes such as *p53* and *PTEN* but also activate the phosphorylation of Cav-1, which is reported to promote cancer cell growth and survival [186]. Tyrosine-14 was documented to be the main phosphorylation site responsible for Cav-1 downstream signaling transduction. Therefore, it is feasible to apply tyrosine kinase inhibitors (TKIs) to interrupt Cav-1 phosphorylation and finally inhibit ROS-induced carcinogenesis. Gefitinib, one kind of TKIs for advanced non-small-cell lung cancer therapy, was reported to inhibit EGF-induced stimulation of both EGFR and downstream Akt and MAPK more efficiently in MCF-7 cells overexpressing Cav-1 than in parental cells [187]. Similarly, the level of phosphorylated Cav-1 was also upregulated in drug-resistant BT474 cells but was blocked by lapatinib [188]. Meanwhile, ovarian cancer cells with high expression of Cav-1 were particularly sensitive to dasatinib [189]. Intriguingly, several TKIs were demonstrated to be effective in preventing carcinogenesis. For example, gefitinib was validated to reduce mammary tumor incidence by 50% [190]. Lapatinib and pazopanib were also demonstrated effective in preventing breast cancer metastasis [191, 192]. What is more is that sorafenib was suggested to be applied for preventing hepatocellular carcinoma recurrence after liver transplantation [193]. All these findings implied that Cav-1 phosphorylation inhibition might be a promising strategy for cancer prevention, and it is of great value to develop Cav-1-targeted TKI for cancer prevention.

The carcinogenic properties of excessive reactive species have prompted the evaluation of dietary antioxidant supplementations as potential cancer preventive agents. However, the preventive efficacies of dietary antioxidant supplementations are disappointing in the current clinical trials. Based on the understanding that mitochondrion is the main source of reactive species in cancer cells, therapies that directly inhibit the production of mitochondrial-derived reactive species, or that scavenge reactive species in mitochondrion, will be more effective than dietary antioxidants, because the latter poorly access the mitochondrial-localized pools of reactive species. Therefore, development of mitochondrion-targeting antioxidants delivery system is a better therapeutic approach for cancer antioxidant prevention. To date, a variety of mitochondrion-targeting antioxidants are being developed, and the best characterized one is 10-(6′-ubiquinonyl) decyltriphenylphosphonium, which could specifically accumulate in the mitochondrial matrix by several hundredfold because of the large mitochondrial transmembrane potential [194]. Caveolae are not the preferred location for Cav-1 in all cell types; instead, Cav-1 can be targeted to a variety of intracellular destinations, particularly in the mitochondrion [21, 22]. *CAV1*-null MEFs (mouse embryonic fibroblasts) had a higher mitochondrial membrane potential and a

preference for glycolysis. Meanwhile, the circulating H_2O_2 and pyruvate levels in $CAV1^{-/-}$ mice were significantly elevated, indicating the altered mitochondrial function and metabolic inflexibility associated with the loss of Cav-1 [195]. Another study also demonstrated that Cav-1 deficiency could increase the mitochondrial membrane condensation, accompanied with dysfunction of respiratory chain efficiency and intrinsic antioxidant defense [196]. Mechanism study further validated that Cav-1 loss resulted in the cytoplasmic and proteasome-dependent degradation of complexes I, III, IV, and V but had no effects on either mitochondrial number or morphology [197]. All these findings suggested that strategies targeting Cav-1 translocation to mitochondrion might be a novel approach for cancer prevention. It is worth to study the detailed molecular mechanisms underlying the targeted delivery of Cav-1 to mitochondrion, and it is anticipated to see enriched mitochondrial Cav-1 expression would decrease the incidence of malignant transformation via inhibiting reactive species overproduction.

7. Conclusions and Perspectives

As stated above, both oxidative stress and Cav-1 are closely connected with the tumorigenesis and progression of cancer. Compared with normal cells, cancer cells usually demonstrate aberrations in oxidative metabolism and signaling pathways, characterized by increased levels of reactive species. Reactive species overproduction could induce tumorigenesis and progression possibly by modulating the expression, degradation, posttranslational modifications (including tyrosine phosphorylation and palmitoylation), and subcellular localization of Cav-1. Meanwhile, Cav-1 mainly acts as a tumor suppressor during cancer initiation and development and also has a feedback regulation effect on oxidative stress. More importantly, emerging evidences have indicated that multiple antioxidants could exert antitumor activities in cancer cells as well as protective activities in normal cells by modulating the Cav-1 pathway. Altogether, these observations suggest that Cav-1 may be a potential oxidative stress-related protein for cancer antioxidant prevention. However, it should be noted that existing studies concerning the interaction between Cav-1 and reactive species were mainly applied in normal endothelial or epithelial cells, but less in cancer cells. Therefore, more research focusing on cancer samples are urgent for a better understanding the role of Cav-1 in oxidative stress-induced cancer initiation and progression, as well as its potential as an oxidative stress-related protein for cancer antioxidant prevention. What is more, development of Cav-1-targeting strategies and antioxidant drugs might shed novel light on cancer prevention and antioxidant research.

In summary, this review reveals the roles of Cav-1 and oxidative stress in tumorigenesis and progression as well as their interaction, proposing Cav-1 as a promising candidate target for cancer antioxidant prevention, possibly providing novel insights for Cav-1-targeted strategies for cancer prevention and treatment.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

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Research Article

Effect of Red Orange Juice Consumption on Body Composition and Nutritional Status in Overweight/Obese Female: A Pilot Study

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The main objective of this research was to determine whether a commercial orange juice rich in anthocyanins could have an effect on body weight and on clinical parameters related to obesity including antioxidant status, lipid profile, and metabolic and inflammatory biomarkers. 11 women with an average BMI of 34.4 ± 4.8 kg/m^2 were enrolled in a pilot study. Over a period of 12 weeks they received 500 mL daily dose into two doses (250 mL) of commercial red orange juice (COJ). The biochemical parameters were measured at baseline and at the end of the study (12 weeks). One month later upon free diet, a follow-up was performed measuring the same variables. The daily consumption of 500 mL of COJ had no significant effects on body weight, while there was a decrease in total cholesterol and LDL cholesterol. The grade of obesity implies different changes in inflammation biomarkers. In obese women, our data do not seem to support evidence that commercial red orange juice consumption acts as functional food preventing obesity and metabolic disorders such as insulin resistance and/or inflammatory status.

1. Introduction

Obesity is well recognized as one of the major public health issues worldwide and its prevalence is increasing [1, 2]. Overweight and/or obesity rates in Italy, like other Western countries, are estimated to be 32% and/or 10%, respectively [3]. Obesity with fat accumulation in the abdominal area, especially in the visceral compartment, may be associated with a higher prevalence of diabetes mellitus, hypertension, dyslipidemia, and coronary heart disease than gluteal-femoral obesity. The specific etiopathogenetic mechanism is not clear, although some evidence suggests that, as a major endocrine and secretory organ, visceral adipose tissue could play a key role in these metabolic complications releasing several hormones, cytokines, and other vasoactive substances [4, 5].

Obesity is commonly caused by eating excessive calories more than energy requirement needs for a long time. As widely known food intake and energy expenditure are regulated by complex interactions between the individual's genetic background and environmental factors [6].

In humans, it is estimated that genes are responsible for only 40% in determining body mass changes while the increased prevalence of obesity in the last 20 years is mainly due to environmental factors in determining high energy intake and low energy expenditure. Lifestyles have become increasingly more sedentary leading to positive energy balance and weight gain.

Recently it was shown that preventing some metabolic disorders associated with obesity, such as diabetes mellitus, was possible by diet and lifestyle modification [7, 8].

In particular, several epidemiological studies confirmed an inverse association between higher fruit and vegetable consumption and the occurrence of cardiovascular disease, cancer, and chronic-degenerative disease [9–13]. Fruits and vegetables supply vitamins, minerals, trace elements, and

dietary fiber and contain a unique combination and amount of compounds called nutraceuticals or phytochemicals with surprising beneficial properties (nutritional and pharmacological). Various phytonutraceuticals with antioxidant properties are able to counteract the inflammatory processes, to delay aging processes and to slow cellular degeneration.

Recently, of great interest is the dietary consumption of the anthocyanins, a water-soluble pigments belonging to flavonoids class. Their most recognized properties are to confer a wide range of colors with red, blue, purple, and violet in many fruits and vegetables and to exert potential health benefits in the free-radical scavenging, antioxidant capacities and antibacterial activity [14–16].

Accumulated research evidence has also highlighted the anthocyanins' role in rats supplemented by anthocyanin-rich diet, in the improvement of blood lipids, ameliorated inflammation, greater decrease in body weight, and better weigh control [17–19].

Few studies were designed to verify the lipid-lowering and/or antioxidative capacity as well as blood glucose and blood pressure control by anthocyanins intake in humans [20–22], while how these bioactive components could be implicated in regulating body weight is not completely clear.

In view of the foregoing considerations, to improve the knowledge and to highlight the health-promoting properties of anthocyanin rich foods, the effect of red orange juice consumption on nutritional anthropometry and on nutritional/metabolic status (blood lipids, vitamin, and hormonal and antioxidant status) in overweight/obese women was studied in a pilot study.

2. Materials and Methods

2.1. Red Orange Juice. A commercial pasteurized red orange juice (COJ) produced by Ortogel (Catania, Italy) was used as the anthocyanins source. Table 1 shows the energy and nutrients provided by 100 mL of COJ as reported by Ortogel. Frozen blood orange juice was given every 15 days to each participant and stored at 4°C before consumption.

2.2. Subjects. The study was conducted in accordance with the Declaration of Helsinki on the human trial performance and informed consent was provided by participants. They were informed about the research purpose and procedure, benefits, and risks, having the freedom to drop out from the study at any time. The study protocol was approved by the Ethical Committee of the San Camillo Forlanini Hospital in Rome. In this study, the mean age of the enrolled women was 36 ± 7 years, while an average BMI of 34.4 ± 4.8 kg/m^2 indicates a presence of obesity. Overweight and/or obese women were recruited from San Camillo Forlanini Hospital and primary care physicians. Volunteers were screened considering the following exclusion criteria: presence of pathological conditions representing a risk for volunteers or likely to influence the outcomes, use of vitamin and/or mineral supplements or drugs in the last 3 months prior to the study, unusual dietary habits (vegetarians, vegans), regular consumption of alcohol > 20 g/day, and smoking habits. Information concerning medical and surgical history and use of drugs or nutritional supplements was collected by a general interview, as well as data on smoking and alcohol consumption. Anthropometric measurements (height and body weight) were performed in the morning in fasting conditions according to the standardized procedure [23]. After this screening, 20 adult women were enrolled, six volunteers declined to participate, three of them dropped out of the study, and the final sample was of 11 subjects.

Table 1: Commercial orange juice: nutritional values per 100 mL*.

Energetic value (kJ)	188
pH	3.6
Total soluble solids (°Brix)	11.2
Proteins (g)	0.6
Fats (g)	0.1
Carbohydrates (g)	10.0
Vitamin C (mg)	55
Anthocyanins (mg)	>50

*Nutrients amount on data fixed by Ortogel.

2.3. Study Design. The 12-week study was a controlled feeding trial conducted by daily intake of blood orange juice. Table 2 summarizes the experimental design. According to literature data and on the basis of juice composition, enrolled subjects drunk 500 mL daily dose (two doses of 250 mL) before mealtime, corresponding to 250 mg of anthocyanins/day. Systolic and diastolic blood pressure and anthropometric measurements were performed before, every four weeks over intervention phase and at the end of COJ consumption. Biochemical parameters were measured at baseline and at the end of the study including the assessment of blood lipids and vitamin and hormonal and antioxidant status. One month later upon free diet, a follow-up was conducted on the same variables. Furthermore, subjects were asked to maintain their usual physical activity, lifestyle, and diet. Monitoring of diet was periodically carried out by 4 days of dietary records collected every 15 days; food intake data were converted into nutrient intake using the Italian food composition tables [24]. Information concerning medical conditions, use of drugs, or nutritional supplements was collected by a general interview, as well as data on lifestyle including smoking and alcohol consumption.

2.4. Methodologies. Anthropometric measurements including body weight, stature, circumferences, and skinfold thicknesses were assessed in accordance with the techniques described by Lohman et al. (1988).

Systolic and diastolic blood pressure was measured in mm Hg using Omron M6 (HEM-7001-E), after subjects had been resting for approximately 8–10 minutes.

Blood samples were collected in EDTA or heparin containing tubes. After centrifuging at 3000 rpm in refrigerate conditions the plasma was obtained and then stored at −80°C until analyses. Aliquots of plasma were used as shown

Table 2: Study design.

	Time →							
	Baseline	2 weeks	4 weeks	6 weeks	8 weeks	10 weeks	12 weeks	Follow-up
Medical examination	x		x		x		x	x
Anthropometric measurements	x		x		x		x	x
Blood withdrawn	x						x	x
Lifestyle questionnaire	x							
Food diary	x	x	x	x	x	x	x	x

below. Plasma Total Antioxidant Capacity was measured using Ferric Reducing Antioxidant Power (FRAP) method [25]; plasma Vitamins A and E determinations were carried out by HPLC as previously described by Maiani et al. [26]. Total ascorbic acid was extracted using the method described by Margolis and Schapira [27] and the quantitative analysis was performed using an HPLC system equipped with a coulometric detector (ESA model 580; Chelmsford, MA, USA) [28]. Total cholesterol, HDL cholesterol, triglycerides, and uric acid concentrations were measured using enzymatic tests (Sentinel Diagnostics, Milano, Italy). LDL cholesterol was estimated by the Friedewald's formula. CRP, TNF-alpha, leptin, adiponectin, and insulin levels were measured by ELISA using commercial kits (DRG International, Inc., BD Biosciences ELISA reagent and Boster Biological Technology, Ltd.). The homeostasis model assessment-estimated insulin resistance (HOMA-IR) was used to assess insulin resistance. HOMA-IR was calculated multiplying fasting plasma insulin (mU/L) by fasting plasma glucose (mg/dL) and then dividing by the constant 405 [29].

2.5. Statistics. The results are presented as means with their standard error. Differences between the end and the start of COJ consumption are given as means and their 95% confidence intervals (95% CIs). All data were tested for normal distribution using the Kolmogorov-Smirnov test. Difference between two phases on same variable was analysed by Paired t-test. Multiple-comparison post hoc correction by Bonferroni was applied. Nonnormal data were analyzed using nonparametric Kruskal-Wallis test. Results were considered significant at $P < 0.05$.

3. Results

No significant changes were observed in body weight on daily consumption of 500 mL of COJ at the end of a 12-week intervention (0.4 kg; 95% CI: −2.7 to 3.96) (Table 4). Figure 1 shows the mean changes in body weight in association to energy intake. Although no significant differences in energy intake were present during the study, the small changes observed in body weight seem to follow the trends in energy intake. Furthermore, after COJ consumption, over one-month follow-up following free diet, an increase in body weight (about 1%), despite decreasing in energy intake, was observed.

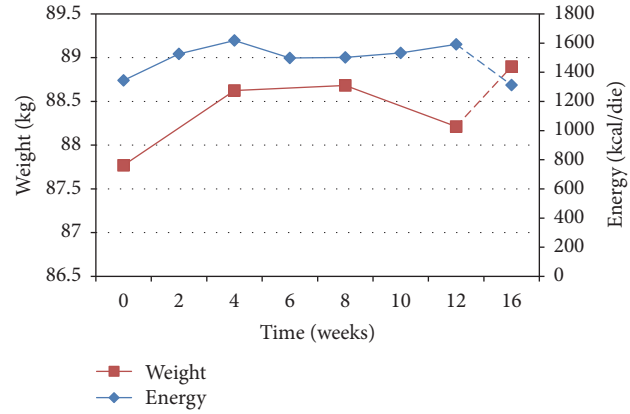

Figure 1: Mean changes in body weight over supplementation phase (12 weeks) and follow-up (one month later) by energy intake (mean ± SE).

Habitual energy, macronutrient, and micronutrient intake over a 12-week supplementation phase and one month later upon free diet (follow-up) are reported in Table 3.

According to dietary survey, no significant differences in habitual diet were observed. However, it should be taken into account that the orange juice consumption led to an increasing daily calories intake (+940 kJ/die), which inevitably affect the body weight, if a decreased energy intake or increased physical activity level are not implemented.

Table 5 displays some biochemical and hemodynamic parameters over COJ consumption (12 weeks) and one month later upon free diet (follow-up). COJ consumption determined a decrease corresponding in a mean value of −11 mg/dL (95% CI: −55 to 7) in total cholesterol levels and a significant decrease in LDL-cholesterol levels −9 mg/dL (95% CI: −30 to 12; $P = 0.046$). Moreover, over one month after COJ consumption (follow-up), moderate increases in total cholesterol (164 ± 14 versus 158 ± 13 mg/dL, resp.), triglycerides (123 ± 20 versus 106 ± 16 mg/dL, resp.), and uric acid levels (4.7 ± 0.4 versus 4.4 ± 0.4 mg/dL, resp.) were highlighted in addition to a significant increase (53.0 ± 8.0 versus 49.4 ± 7.5 μg/dL, resp.; $P = 0.04$) in vitamin A and a decrease (1.24±0.16 *versus* 1.28±0.16 mg/dL, resp; $P = 0.007$) in vitamin E. Moreover a significant decrease in diastolic blood pressure (74 ± 2 versus 83 ± 3 mmHg, resp.; $P = 0.01$) was evidenced over follow-up. Measures of total ascorbic

Table 3: Habitual energy, macronutrients, and micronutrient intake over supplementation phase (12 weeks) and one month later (follow-up).

	Before COJ	After COJ	Follow-up
Energy (kJ/die)	5623 ± 370	5704 ± 37	5489 ± 426
Proteins (g/die)	64 ± 4	55 ± 3	56 ± 2
Lipids (g/die)	58 ± 4	56 ± 8	54 ± 5
PUFA (g/die)	5.8 ± 1.6	5.2 ± 1.9	4.9 ± 1.0
MUFA (g/die)	8.7 ± 1.2	9.2 ± 2.0	8.5 ± 1.0
SFA (g/die)	14.7 ± 1.2	14.8 ± 1.7	12.8 ± 1.4
Carbohydrates (g/die)	145 ± 17	165 ± 11	154 ± 14
Anthocyanins (mg/die)	52 ± 8	41 ± 5	35 ± 6
Ascorbic acid (mg/die)	78 ± 17	62 ± 11	67 ± 8
Fibra	11.6 ± 1.3	11.0 ± 1.0	11.9 ± 0.4
Iron (mg/die)	6.75 ± 0.49	6.53 ± 1.47	6.82 ± 1.40
Calcium (mg/die)	406 ± 41	324 ± 50	422 ± 46
Retinol eq. (μg/die)	714 ± 101	686 ± 114	916 ± 201
α-Tocopherol (mg/die)	8.51 ± 0.88	8.94 ± 1.15	9.12 ± 0.87

Data are expressed as mean ± SE.
No statistical difference between phases.

Table 4: Changes in body composition following consumption of 500 mL of COJ.

	Before COJ	After COJ	P	Δ^a (95% CI)
Body weight (kg)	87.8 ± 4.5	88.9 ± 4.5	n.s.	0.4 (−2.7 to 3.96)
BMI (kg/m^2)	34.4 ± 1.4	34.6 ± 1.5	n.s.	0.2 (−1.2 to 1.7)
Waist circumference (cm)	96.0 ± 2.9	96.0 ± 3.0	n.s.	−0.0 (−5.6 to 6.3)
Hip circumference (cm)	119.38 ± 3.0	119.06 ± 3.16	n.s.	−0.3 (−4.2 to 3.0)
WHR	0.807 ± 0.02	0.808 ± 0.02	n.s.	0.007 (−0.011 to 0.06)

Data are expressed as mean ± SEM; n.s., not significant.
ANOVA: after COJ versus before COJ.
Δ^a: differences from after to before COJ (mean values and their 95% confidence intervals).
WHR: waist/hip circumferences.

Table 5: Biochemical and hemodynamic parameters over 12 weeks following COJ consumption and one month later on free diet (follow-up).

	Before COJ	After COJ	P	Δ^a (95% CI)	Follow up	P
Systolic pressure (mmHg)	125 ± 5	131 ± 5	n.s.	6 (−15 to 16)	122 ± 4	n.s.
Diastolic Pressure (mmHg)	81 ± 2	83 ± 3	n.s.	3 (−10 to 10)	74 ± 2	0.01
FRAP (μmolFe^{2+}/L)	832 ± 45	827 ± 42	n.s.	−5 (−93 to 116)	841 ± 61	n.s.
Uric acid (mg/dL)	4.6 ± 0.4	4.4 ± 0.4	n.s.	−0.2 (−0.9 to 1.0)	4.7 ± 0.4	n.s.
Total cholesterol (mg/dL)	170 ± 14	158 ± 13	n.s.	−11 (−55 to 7)	164 ± 14	n.s.
Triglycerides (mg/dL)	104 ± 21	106 ± 16	n.s.	3 (−108 to 89)	123 ± 20	n.s.
HDL-cholesterol (mg/dL)	66 ± 3	63 ± 2	n.s.	−3 (−15 to 4)	65 ± 2	n.s.
LDL-cholesterol (mg/dL)	83 ± 12	74 ± 13	0.046	−9 (−30 to 12)	74 ± 13	n.s.
Vitamin A (μg/dL)	52.1 ± 8.3	49.4 ± 7.5	n.s.	−2.7 (−22.5 to 7.6)	53.0 ± 8.0	0.04
Vitamin E (mg/dL)	1.32 ± 0.16	1.28 ± 0.16	n.s.	0.08 (−0.40 to 0.33)	1.24 ± 0.16	0.007
Vitamin C (mg/dL)	1.01 ± 0.05	1.18 ± 0.08	n.s.	0.17 (−0.16 to 0.62)	1.06 ± 0.09	n.s.
AA (mg/dL)	0.59 ± 0.07	0.64 ± 0.12	n.s.	0.06 (−0.42 to 0.69)	0.52 ± 0.07	n.s
DHA (mg/dL)	0.42 ± 0.08	0.53 ± 0.07	n.s.	0.115 (−0.52 to 0.44)	0.54 ± 0.07	n.s

Data are expressed as mean ± SEM; n.s., not significant.
AA: ascorbic acid; DHA: dehydroascorbic acid.
P: ANOVA.
Δ^a: differences from after to before COJ (mean values and their 95% confidence intervals).

Table 6: Metabolic and inflammatory biomarkers over supplementation phase (12 weeks) and follow-up (one month later).

	Before COJ	After COJ	P	Δ^a (95% CI)	Follow-up	P
Leptin (mg/dL)	29.0 ± 4.4	32.6 ± 4.1	n.s.	3.6 (−12.1 to 20.6)	31.2 ± 4.2	n.s.
Adiponectin (μg/mL)	9.54 ± 1.33	7.90 ± 1.08	n.s.	−1.6 (−8.5 to 4.3)	7.66 ± 1.06	n.s.
Glucose (mg/dL)	100.9 ± 1.9	105.7 ± 3.3	0.07	4.8 (−5.6 to 20.5)	105.7 ± 2.7	n.s.
Insulin (mU/L)	10.6 ± 1.7	12.2 ± 2.1	n.s.	1.7 (−2.3 to 15.2)	11.4 ± 1.6	n.s.
HOMA-IR	2.61 ± 1.27	3.16 ± 0.54	n.s	0.6 (−0.5 to 4.1)	3.00 ± 0.47	n.s.
CRP (μg/mL)	4.45 ± 1.39	5.91 ± 1.85	n.s	1.5 (−1.2 to 6.5)	6.10 ± 1.73	n.s
TNF-α (pg/mL)	33.24 ± 3.10	33.69 ± 2.69	n.s	0.4 (−10.7 to 20.4)	37.58 ± 2.03	n.s

Data are expressed as mean ± SEM; n.s., not significant.
P: ANOVA.
Δ^a: differences from after to before COJ (mean values and their 95% confidence intervals).

acid (1.18 ± 0.08 mg/dL), ascorbate (0.64 ± 0.12 mg/dL), and dehydroascorbate (0.53 ± 0.07 mg/dL) were higher after COJ consumption respect baseline (1.01 ± 0.05, 0.59 ± 0.07, and 0.42 ± 0.08 mg/dL, resp.).

Metabolic and inflammatory biomarkers over 12 weeks of COJ consumption and follow-up are presented in Table 6. A mean increase of 4.8 mg/dL (95% CI: −5.6 to 20.5) in glucose levels compared to baseline was present (105.7 ± 3.3 versus 100.9 ± 1.9 mg/dL, resp.). Furthermore, higher HOMA-IR of 0.6 (95% CI: −0.5 to 4.1) and higher leptin of 3.6 mg/dL (95% CI: −12.1 to 20.6) with lower adiponectin of −1.6 μg/mL (95% CI: −8.5 to 4.3) levels were associated with consumption of COJ. In addition, an increased inflammatory response was present upon consumption of COJ.

Whereas no relationship was demonstrated between several parameters and BMI, to explore the possible influence of BMI on some variables, a further assessment was performed by distributing the results obtained at the end of supplementation by BMI classes. Indeed [30], as reported in Figure 2, a clear relationship between body mass classes (<30, 30–35, >35 kg/m^2) and CRP increments with respect to baseline was relieved (−0.62 ± 0.62 μg/mL, −0.10 ± 0.36 μg/mL, and 4.46±1.09 μg/mL, resp.). Similar trend was present for TNF-α increments −6.38 ± 1.60 pg/mL, 1.01 ± 1.32 pg/mL, and 4.07 ± 1.48 pg/mL, respectively, by BMI classes. Moreover, Figure 3 reports the pattern of changes by BMI classes over 12-week COJ consumption in leptin and adiponectin levels. Subjects with the highest BMI classes showed a lower increase in leptin (37.4 ± 6.5 versus 35.2 ± 6.541; 34.4 ± 5.9 versus 30.2 ± 5.9; 18.3 ± 9.3 versus 13.6 ± 9.3, resp., for BMI <30, 30–35, and >35 kg/m^2) as well as lower decrease in adiponectin levels.

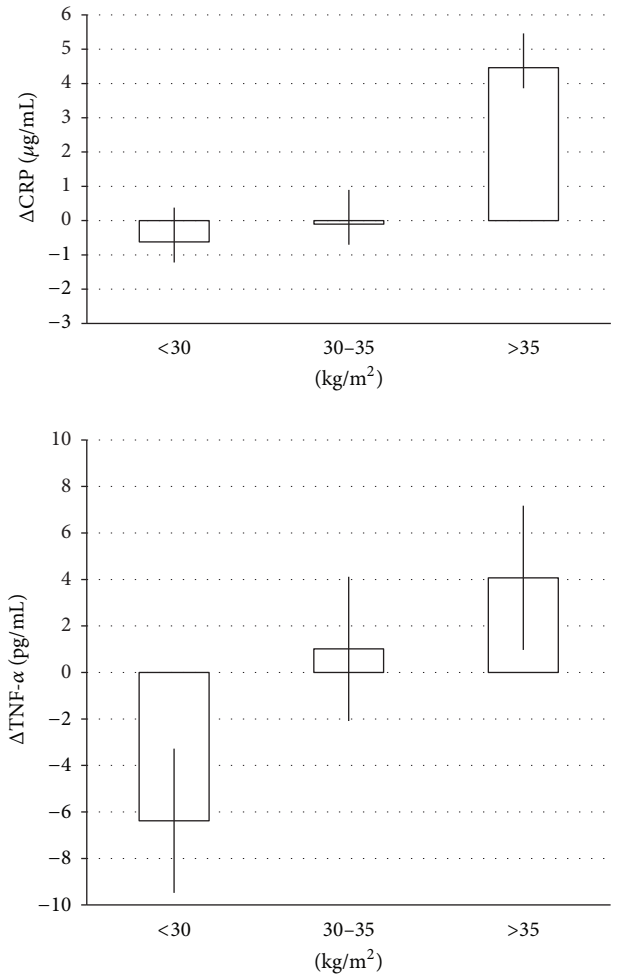

Figure 2: Pattern of change in CRP (μg/mL) and TNF-α (pg/mL) by BMI levels.

4. Discussion

There is growing scientific interest in the nutraceuticals and functional foods and their potential protective effects on human health, both in prevention and in treatment of obesity and cardiovascular diseases. The orange juice contains a set of powerful bioactive molecules including flavonoids, carotenoids, vitamin C, folate, and other phytochemical compounds. It is also well known that COJ contains a high concentration of anthocyanins particularly cyanidin-3-glucoside, which seems to be implicated in the reduction of body weight and fat accumulation. The effect of COJ consumption on weight gain is controversial, especially because

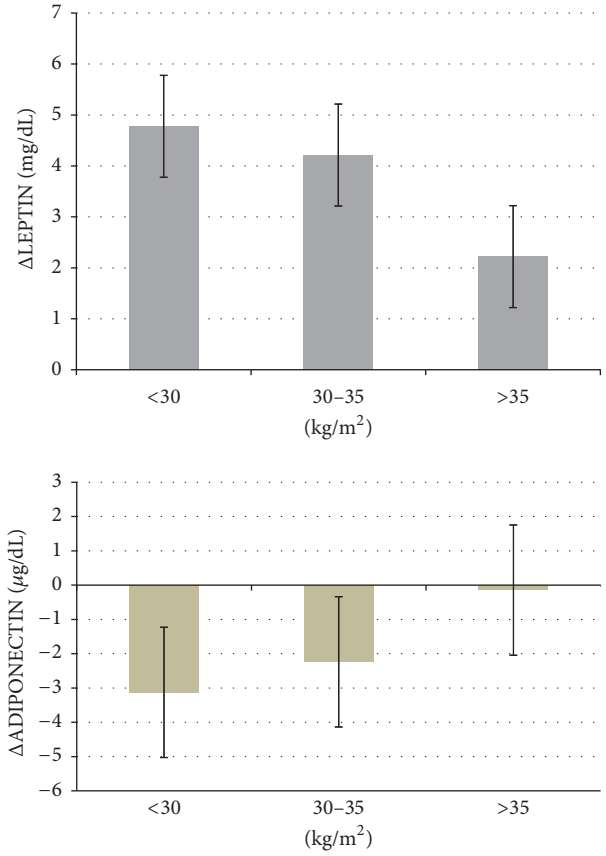

Figure 3: Pattern of change in leptin (mg/dL) and adiponectin (μg/dL) by BMI levels.

of its sugar content [31, 32]. In this study, COJ consumption resulted in augmented carbohydrates intakes with respect to baseline and one month later on free diet (follow-up). Experimental studies in animal models have shown a probable role of anthocyanins to attenuate obesity producing a decreased weight and adipose tissue and ameliorate insulin resistance [19, 33–35]. Previous studies on humans [36, 37] did not find significant anthropometric changes after regular orange juice consumption. Basile et al. [38] found a significant reduction in waist circumference in women over 8 weeks following COJ consumption but not in men. In addition, despite the higher fat intake among women, which could increase total cholesterol and LDL-cholesterol, there were important changes/improvement in their lipid profile. Same effect was reported by Dourado and Cesar [37]; an improvement of the lipid profile, evidenced by a reduction in total cholesterol and LDL-cholesterol in overweight subjects following COJ consumption, was observed, even if significant decreased lipids consumption was present. Total cholesterol and LDL-cholesterol levels decreased in our sample with slightly increase in dietary lipids, while triglycerides and HDL-cholesterol were unchanged. The absence in reducing triglyceride levels seems to confirm the primary effect due to gender, fasting glucose, insulin levels, insulin resistance, and total fructose intake [39]. In addition we failed to detect a raising HDL-cholesterol levels after COJ consumption was found in previous studies on humans [37, 38, 40]. Only two overweight subjects exhibited an increase of 3% with respect to HDL-cholesterol levels after COJ consumption (70 ± 4 versus 68 ± 4 mg/dL), while a mean reduction of 5% was present in subjects with a BMI > 30 kg/m^2 (63.5 ± 3 versus 66.5 ± 3 kg/m^2) (data not shown). These different outcomes may be explained by type of administration (fresh and/or commercial product or pure compound), dose, length of the study, and different sizes and clinical characteristics of the enrolled sample. In agreement with several studies, other components rather than or in addition to anthocyanins may contribute to enhancing lipid profile and to exert beneficial antiobesity effects [36, 41]. As it is well known, the obesity corresponds to a subclinical inflammatory condition that promotes the production of proinflammatory factors involved in the pathogenesis of insulin resistance [42]. Our sample at baseline was characterized by overproduction of proinflammatory mediators, such as circulating levels of C-reactive protein (4.45 ± 1.39 μg/mL), TNF-α (33.24 ± 3.10 pg/mL), and insulin resistance (HOMA-IR 2.61 ± 1.27) and normal lipid profile, several of which are risk factors for cardiovascular diseases, metabolic syndrome, and diabetes. The only two overweight subjects showed a mean decrease of 34% and 23%, respectively, for CRP and TNF-α levels, while, among women with obesity (BMI > 30 kg/m^2), mean increase of 39% and 5% was present, respectively. Thus, different grade of obesity implied different changes in inflammation biomarkers. The increase in blood glucose observed at 12 weeks after supplementation (105.7 ± 3.3 mg/dL) compared to baseline (100.9 ± 1.9 mg/dL) could be attributed to sugars contained in COJ (50 g/day), which may have influenced the plasma glucose levels as well as body weight (88.9 ± 4.9 versus 87.8 ± 4.5 kg). Factors associated with diet of subjects should be excluded because there were no significant changes throughout the study. In spite of increases of the ascorbate and dehydroascorbate levels, COJ consumption causes an increase of the DHA/AA ratio (83%) compared to baseline (72%). As reported [43] higher ratio may relate to the varied metabolic roles of the vitamin instead of inadequate dietary vitamin C intake. On the other hand, the decrease of uric acid and vitamin E seem to justify the decline in the levels of FRAP which contribute to the 60% and 5%, respectively [25].

Li et al. [44] have demonstrated that anthocyanin supplementation exerts beneficial metabolic effects in subjects with type 2 diabetes by improving dyslipidemia, enhancing antioxidant capacity, and preventing insulin resistance. Asgary et al. [45] have reported beneficial effects on the physiological characteristics of healthy volunteers particularly in some of inflammatory markers after the consumption of commercial and fresh orange juice. In our study, COJ consumption in obese women was associated with increased metabolic abnormalities associated with insulin resistance; only two overweight subjects showed a HOMA-IR value < 2.2 associated with a reduction of 14% after 12 weeks of COJ consumption. Obese women (>30 kg/m^2) showed a mean increase of 39% in HOMA-IR value at the end of supplementation phase (4.2 ±

0.6 versus 3.5 ± 0.6). Higher plasma leptin concentrations (32.4 ± 4.1 versus 29.0 ± 4.4 mg/dL) and lower adiponectin levels (9.54 ± 1.33 versus 7.90 ± 1.08 μg/dL) were highlighted suggesting increased obesity-related complications. In fact, proinflammatory mediators such as TNF-α and CRP slightly increased, as well as a decreased antioxidant status including FRAP, uric acid, vitamin A, and vitamin E which is evidenced. As reported by Fantuzzi [5], the mechanism by which the insulin-resistant state is associated with low levels of adiponectin is not clear while its anti-inflammatory role appears confirmed. Adiponectin reduces the production and activity of TNF-α; in contrast, in obese subjects increased TNF-α production could explain the decline of adiponectin levels. Hajri et al. [46] have reported that reduced insulin stimulation and increased TNF-α production are among the factors that contribute to the decline of adiponectin production and alteration of isomer composition in obese insulin-resistant subjects. Furthermore, some evidence indicates that TNF-α is an important player in the state of insulin resistance observed during obesity by interfering with insulin signaling [47]. Castro et al. [48] highlighted a probable pathophysiological and molecular mechanisms involved in the link between increased visceral adipose tissue, insulin resistance, and comorbidities. The mechanisms involved in the etiopathogenesis of insulin resistance related to obesity occur due to prereceptor, receptor, and/or postreceptor impairments, mainly to insulin receptor downregulation secondary to hyperinsulinemia (receptor) and inhibition of the intracellular cascades by several adiposity-related factors (e.g., impaired adipokines and/or cytokines secretion) (after receptor) [49]. At last, our data support the evidenced strong relationship between leptin and adiponectin as well as their modulation by BMI and dietary pattern diet; the linkage between leptin resistance and obesity was confirmed, too.

5. Conclusion

Although our research is a pilot study at low subjects stratifying by BMI classes and our findings cannot be generalized, they might be helpful in planning further studies design and supplementary knowledge.

In obese women our data do not seem to support evidence that COJ consumption acts as functional food and could be consumed as part of a healthy diet to prevent obesity and metabolic disorders such as insulin resistance and/or inflammatory status. We confirm the role of adipose tissue as endocrine and secretory organ in releasing adipokines and proinflammatory molecules. The grade of obesity implies different changes in inflammation biomarkers. Our findings support the hypothesis that COJ consumption was significantly associated with favorable effects on total cholesterol and LDL-cholesterol levels in subjects with normal lipid profile.

In the light of the aforementioned considerations and results we suggest the consumption of freshly squeezed orange juice, without added sugars in obese women as part of a controlled diet or alternatively taking into account the global energy diet. The combination with increasing physical activity for maintaining a healthy body weight through healthier food choices could improve the comorbidities related to the obesity.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this paper.

Authors' Contributions

All authors have made substantial contribution to this work. All authors have read and approved the final paper.

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Research Article

Antioxidant and *Ex Vivo* Immune System Regulatory Properties of *Boswellia serrata* Extracts

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Boswellia serrata (BS) is an important traditional medicinal plant that currently represents an interesting topic for pharmaceutical research since it possesses several pharmacological properties (e.g., anti-inflammatory, antimicrobial, and antitumour). The safety and versatility of this dietary supplement should allow for its use in numerous pathological conditions; however the quality of the extracts needs to be standardized to increase the clinical success rate resulting from its use. In the present study, different commercially available *B. serrata* extracts were employed to compare their AKBA content and in vitro antioxidant power. Furthermore, their ability to modulate the immune system regulatory properties was investigated. Our results showed that the AKBA content varied from 3.83 ± 0.10 to 0.03 ± 0.004%, with one sample in which it was not detectable. The highest antioxidant power and phenolic content were shown by the same extract, which also exhibited the highest AKBA concentration. Finally, the BS extracts showed the ability to influence the regulatory and effector T-cell compartments. Our results suggest that frankincense should be further investigated for its promising potentiality to modulate not only inflammation/oxidative stress but also immune dysregulation, but attention should be paid to the composition of the commercial extracts.

1. Introduction

The gum resin of *Boswellia serrata* (BS), a traditional treatment of Ayurvedic medicine in India also identified as Indian frankincense, Salai Guggal, or Indian olibanum, has been used for centuries as a remedy for many health problems [1].

Indeed, the anti-inflammatory, antiarthrogenic, and analgesic activities of its dried resinous gum (guggulu), derived from tapping the *Boswellia* tree, have been recognized since ancient times [2]. The inflammatory response represents the first-line defense of the body to tissue damage and/or to microbial invasion and it determines the recruitment of immune cells and some plasma proteins [3]. The final goal of inflammation is healing, elimination of the external or internal inflammation *noxae,* and the restoration of homeostasis.

This immune response should be self-limiting but the persistence of the stimulus in predisposed subjects leads to chronification of the process and eventually to irreversible tissue injury. Indeed, persisting low-grade inflammation plays a key role in the pathogenesis of many chronic diseases [4] and most of these diseases are also associated with increased production of reactive oxygen species (ROS), which results in oxidative stress [5]. Therefore, inflammation is tightly linked with oxidative stress [6] by an interdependent relationship and both participate in the pathogenesis of many chronic diseases [4].

During recent decades, many authors have investigated the mechanisms of action of BS extracts related to the inflammatory process. Studies in animal models showed that the ingestion of a defatted alcoholic extract of *Boswellia* decreased polymorphonuclear leukocyte infiltration and migration as well as primary antibody synthesis [7, 8] and led to almost total inhibition of the classical complement pathway [9]. In vitro studies revealed that the boswellic acids, a group

of pentacyclic triterpenoid compounds, and their acetylated derivatives inhibit the biosynthesis of leukotrienes, the proinflammatory 5-lipoxygenase products which cause increased permeability [10], in a dose dependent manner [11]. In addition, Cuaz-Pérolin et al. [12] observed that 3-acetyl-11-keto-beta-boswellic acid (AKBA) was a natural inhibitor of the transcription factor NFkB, whose presence is a prerequisite for the formation/action of cytokines/chemokines involved in inflammatory reactions.

Therefore, these natural compounds can dampen the inflammatory response, but also simultaneously reduce oxidative stress, as observed by Umar et al. [13].

In recent years, extracts from the gum resin of BS have been shown to target both the humoral and adaptive immune responses [14] eventually interfering with the inflammatory cascade [15].

However, to the best of our knowledge, no studies have yet investigated whether the BS extracts can exert any effects on specific T-cell subsets whose balance is crucial for the maintenance of immune homeostasis: the regulatory T-cells (Tregs) and the proinflammatory Th1/Th17 cells. In the present study, different commercially available *B. serrata* extracts were employed as follows: (i) to compare their composition and in vitro antioxidant power; (ii) to test their ability to modulate Treg/Th1/Th17 cells *ex vivo*.

2. Materials and Methods

2.1. Chemicals and Plant Material. All chemicals used were of analytical reagent grade from Sigma-Aldrich (St. Louis, MO, USA). Six (A, B, C, D, E, and F, resp.) of the seven BS oleogum resins utilized in the present study were commercially available and were certified for a content of boswellic acids of 65%. The pale yellow or white amorphous powders were insoluble in water but soluble in methanol and dimethyl sulfoxide (DMSO). The seventh (G) BS extract was an aqueous extract obtained by a process of bioliquefaction based on enzyme biocatalysis [16] and was kindly offered by its producer (Phenbiox, Calderara di Reno, Bologna, Italy).

2.2. TLC Analysis. BS extract separation was performed on 20 × 20 cm silica gel plates with a fluorescent indicator at 254 nm (Sigma-Aldrich, St. Louis, MO, USA). Pentane and diethyl ether (2 : 1) containing 1% (v/v) of acetic acid were used as a mobile phase. Twenty mg of BS extracts was dissolved in 300 μL of ethanol, sonicated for 5 minutes, and centrifuged. Clear supernatant (10 μL) was carefully layered at 1.5 cm from the bottom of the plate giving an elution distance of 9 cm. After the separation, plates were observed at 254 nm and developed with anisaldehyde (5 mL) in glacial acetic acid (50 mL) and H_2SO_4 (1 mL). The TLC analysis of BS G extract was not performed due to its particular formulation.

2.3. HPLC-DAD Analysis. A HPLC system (Beckman Coulter, Brea, CA, USA), comprising a 116 pump, a 507 automatic autosampler, an UV-Diode Array 168 detector, and integration software 32 Karat, was used for the analysis of seven BS extracts. Samples were prepared by dissolving extracts in methanol. Briefly, the separation was performed using a reverse phase column Luna C18 5 μm 250 × 4.6 mm (Phenomenex, Torrance, CA, USA) with a guard column PR C-18 5 μm 15 × 4.6 mm (Phenomenex, Torrance, CA, USA). Chromatographic separation was achieved in isocratic conditions at room temperature. The mobile phase was a mixture of phosphoric acid (H_3PO_4 10 mM in water) and acetonitrile (19 : 81 v/v). The flow rate was 1 mL/min, and the injection volume was 50 μL. The analyses were made at two different wavelengths (210 and 260 nm) and UV spectra were recorded in the range of 190–300 nm. A standard stock solution was prepared by dissolving 5 mg of AKBA analytical standard (Sigma-Aldrich, 5 mg, batch number BCBN2928V, CAS number 416619) in methanol (5 mL). The calibration curve was obtained by analyzing nine serial dilutions (50 ppm, 25 ppm, 15 ppm, 10 ppm, 7.5 ppm, 5 ppm, 2.5 ppm, 1 ppm, and 0.5 ppm) of the stock solution and by plotting the peak area measured at 260 nm against AKBA concentrations. The following equation of the curve was obtained:

$$y = 79739x - 5414, \quad R^2 = 0.999. \tag{1}$$

The AKBA peaks were identified on the basis of the retention time on the chromatogram at 260 nm. All measurements were performed in triplicate and data were reported as mean ± SD.

2.4. Quantification of Total Phenolic Content (Folin-Ciocalteu Method). The total content of polyphenolics was determined by a colorimetric method as described by Singleton and Rossi [17] and adapted to a 96-well plate format. Briefly, the seven BS extracts were redissolved in 1 mL of methanol and 100 μL/well of each extract was dispensed into a flat bottom 96-well tissue culture plate (Becton Dickinson, Lincoln Park, NJ); then 150 μL Folin-Ciocalteu reagent (1 mL Folin-Denis' reagent in 4 mL H_2O) was added. The plate was incubated for 10 min at 37°C. Next, 50 μL of a saturated Na_2CO_3 solution in H_2O was added to each well and the plate was incubated for a further 10 min. The absorbance was measured at 765 nm. A standard calibration curve was plotted using gallic acid (0–300 mg/L). The results were expressed as g of gallic acid equivalents (GAE) per g of dry weight of BS extract. The results were expressed as the average of three measurements.

2.5. Antioxidant Activity. Free radical scavenging activity was studied using 1,1-diphenyl-2-picrylhydrazyl (DPPH) on a microplate analytical assay according to the procedures described by Srinivasan et al. [18], while the total radical scavenging capacity of the same products was measured by the 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay as modified by Re et al. [19], for application to a 96-well microplate assay.

Finally, the determination of antioxidant activity by FRAP assay was carried out according to the procedure described by Müller et al. [20], monitoring the reduction of $Fe3^+$-tripyridyl triazine (TPTZ) to blue-colored $Fe2^+$-TPTZ.

Trolox was used as standard in all assays and the ability of BS extracts to scavenge the different radicals was expressed as tocopherol-equivalent antioxidant capacity (mmol TE/g of product) and, for DPPH and ABTS assays, also as IC_{50}, the latter defined as the concentration of the tested material required to cause a 50% decrease in initial DPPH/ABTS concentration. All measurements were performed in triplicate and reported as mean ± standard deviation (SD).

2.6. Immune Responses

2.6.1. Cell Proliferation Assay. Human peripheral blood mononuclear cells (PBMCs) of seven healthy donors (HD; 5 male and 2 female, age mean ± standard deviation: 47 ± 12.1 years) were isolated from fresh heparinized venous blood (10 mL/HD) by gradient separation. The study was approved by the local ethics committee (CEAS Umbria) and written informed consent was obtained from participants in accordance with the Declaration of Helsinki. The final concentration of live cells was adjusted to 1×10^6/mL in complete medium (RPMI-1640 medium containing 10% heat-inactivated serum, L-glutamine (2 mM), Euroclone®, penicillin (100 U/mL), and streptomycin (100 μg/mL), Biochrom^{AG}, Berlin). PBMCs were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) cell tracer (BioLegend, San Diego, CA), dispensed into flat bottom 24-well tissue culture plates (Becton Dickinson, Lincoln Park, NJ) (1 mL/well), and cultured for 5 days at 37°C in 5% CO_2. For proliferation stimuli were either 1 μg/mL of pokeweed mitogen (PWM; Sigma-Aldrich Co. Ltd., Saint Louis, Missouri) or 1.2 μg/mL of phytohemagglutinin (PHA; Biochrom^{AG}, Berlin), in the presence or absence of two BS extracts (0.1 μg/mL). BS extract A revealed the highest in vitro antioxidant power; BS extract G was obtained with a different extraction method compared to other BS compounds (by Phenbiox srl). So lymphocytes were exposed, in the culture medium, to an AKBA concentration of 3.8 ng/mL that resulted below the mean maximal concentration of 6 ng/mL detected in human plasma after an oral administration of a BS dry extract in fasted condition [21].

A negative control was represented by PBMC cultured without any mitogen/extract (C), so that the base proliferation could be estimated [22]. Therefore, nine different experimental theses for each blood sample were tested. Flow cytometry analyses were performed on a standard FACSCalibur™ flow cytometer (Becton Dickinson, Mountain View, CA) running the CellQuestPro™ software. The results of the lymphocyte proliferation assay were expressed as a percentage (%). Furthermore, the lymphocyte proliferation index (LPI) was calculated with the following formula:

$$\text{LPI} = \frac{(\text{FP} - \text{BV})}{\text{BV}} * 100, \quad (2)$$

where the FP values are represented by the "final percentages" of cell proliferation (after 5 days in culture with BSs and with/without the mitogens), whereas the BV values are represented by the "basal values" obtained by cells either stimulated (with PHA and PWM) or not (CTR) with the mitogen but without BSs [23].

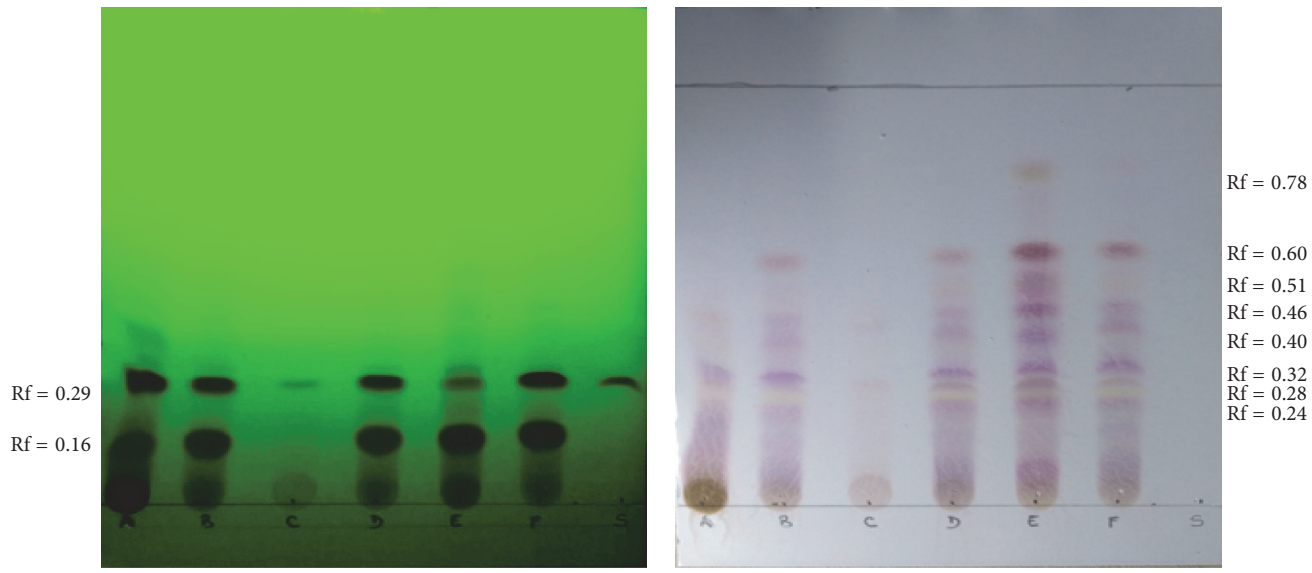
2.6.2. Phenotypic Characterization of Peripheral Blood Mononuclear Cells. PBMCs obtained from the seven volunteers were seeded further (1×10^6 cells/well) into additional flat bottom 24-well tissue culture plates and cultured, with or without PHA, for 5 days at 37°C in 5% CO_2. After culture, six-hour in vitro stimulation with 25 ng/mL phorbol 12-myristate 13-acetate (PMA), 1 μg/mL ionomycin, and 1 μL/mL BD GolgiPlug™ (BD Biosciences) in complete medium was performed. For surface staining, fluorescein isothiocyanate (FITC), PeCy7, or APC labelled antihuman CD4, CD3, and CD25 and respective isotypes were used (BD Biosciences, San Jose, CA, USA, Immunotools). Then, cells were permeabilised with 0.1% saponin blocking buffer after 4% paraformaldehyde fixation to perform intracellular staining with Alexa Fluor 647 or Phycoerythrin (PE) antihuman IL-17 and INFγ, and their isotype controls were used (BD Biosciences). When required, cells were permeabilised with commercially available Forkhead box protein P3 stain buffer (BD Biosciences) for intracellular staining with PE-labelled mAb to human FoxP3 and respective isotype controls [24]. Debris was excluded by backgating to CD3 T-cells in forward scatter/side scatter (FSC/SSC) plots. Samples were analyzed using FACSCalibur flow cytometer (BD) and CellQuestPro software (BD).

2.7. Statistical Analysis. The results of immune responses are reported as mean ± standard error of the mean (SEM) from seven samples of different HD. The unpaired Student's t-test was used to compare biological data from controls with that from BS A or BS G treated samples, respectively (GraphPad Prism, 2007) [25]. p values < 0.05 for two-tailed test were considered statistically significant.

3. Results

3.1. TLC Chromatograms. The preliminary qualitative TLC screening of the BS extracts is shown in Figure 1. The UV analysis revealed two main spots visible in all samples, with the exception of extract C. The first spot had a Rf of 0.16, whereas the second spot had a Rf of 0.29. The use of the AKBA standard (lane S) allowed for the identification of this boswellic acid in the spot with Rf of 0.29. It is noticeable that extract C lacked the first spot and presented only traces of the spot corresponding to AKBA, while extract A presented the greatest spot referring to AKBA. Extracts A and E presented other components absorbing at 254 nm. Figure 1(b) demonstrates the pattern obtained after dyeing with anisaldehyde, heating, and color development. Similar profiles were obtained for extracts B, D, E, and F. Instead, extract E was characterized by a major number of spots; extract A lost the spots at the higher Rf; and extract C was found to have fewer components. The majority of samples shared the spots detected at Rfs of 0.24, 0.32, 0.40, 0.46, and 0.60.

3.2. HPLC-DAD Analysis. At 260 nm, the majority of extracts presented two major peaks: the first one, at Rt of 13.2 min, and



(a) (b)

Figure 1: UV detection at 254 nm (a) for UV-active boswellic acids. Chromatograms after dyeing with anisaldehyde (b). Rf values are reported for the most relevant spots. A–F: six different powder extracts of *Boswellia serrata* gum resin; S: 3-acetyl-11-keto-beta-boswellic-acid (AKBA) analytical standard (Rf = 0.29).

Table 1: AKBA quantification in *Boswellia serrata* extracts. Data are reported as mean ± SD (n = 3). Samples A–F = powder extracts, sample G = hydroenzymatic extract.

Sample	Concentration[§]	% in *B. serrata* extract[#]
A	38.30 ± 1.01	3.83 ± 0.10
B	17.18 ± 0.05	1.72 ± 0.005
C	3.08 ± 0.06	0.31 ± 0.01
D	24.35 ± 1.87	2.43 ± 0.19
E	nd[*]	nd
F	21.07 ± 0.16	2.11 ± 0.02
G	0.29 ± 0.04	0.03 ± 0.004

[§]AKBA concentration is expressed as mg/g of powder extract, with the exception of sample G (mg/mL of hydroenzymatic extract); [#]AKBA percentage is expressed as g/100 g of powder extract, with the exception of sample G (g/100 mL of hydroenzymatic extract); [*]nd = not detectable.

the second one, identified as AKBA by the use of an analytical standard, at Rt of 26 min. Other minor peaks were also present. AKBA concentrations for each sample, calculated on the basis of the peak area and the calibration curve, are shown in Table 1. Extract A presented significantly higher amounts of AKBA as compared with the other samples. Extract C presented only a small peak of AKBA and was lacking the first peak.

Other components in the BS extracts were visualized at 210 nm. The chromatograms of all the BS extracts analyzed at the different wavelengths to highlight the variability of the components present in the samples are reported in Figure 2.

3.3. Determination of Total Phenolic Content and Antioxidant Capacity of Boswellia serrata Extracts. All the BS extracts utilized in the present study exhibited a relatively low content in phenolics ranging from 7.68 ± 0.9 mg gallic acid equivalent (GAE)/g (extract A) to 0.11 ± 0.05 mg GAE/g (aqueous extract G) (Table 2). The chemical complexity of the extracts, often mixtures of many compounds with differences in functional groups, polarity, and chemical behavior, could lead to scattered results, depending on the antioxidant test employed. For these reasons, in the present study, the BS extracts were screened for their free radical scavenging and reducing properties through three test systems: (a) 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, (b) monitoring of the reduction power of $Fe3^{+}$ (FRAP assay), and (c) evaluating the total radical scavenging capacity (ABTS assay). All the BS extracts analyzed showed relative radical scavenging activities in all the assays employed, revealing antioxidant powers lower (from 34 to nearly 580 times) than that of Trolox (positive control, Table 2).

However, between the BS extracts investigated, the BS extract A showed the highest scavenging reducing power and the highest polyphenolic content.

3.4. Immunomodulatory Activity. The in vitro lymphocyte proliferation (CFSE assay) was not influenced by the BS extracts if cells were cultured without any activator (data not shown) or if stimulated by PHA. However, when cells were activated by PWM, the addition of BS extracts induced a significantly higher lymphocyte response (Figures 3(a) and 3(b)). No significant differences were observed between the two types of BSs for the LPI (Figures 3(c) and 3(d)).

The in vitro regulatory or Th1/Th17 proinflammatory responses (Figures 4(d), 4(e), and 4(f)) were not significantly modulated by the addition of the BS extracts when PBMCs were triggered by PHA. Neither type of utilized extract (A versus G) elicited an altered response.

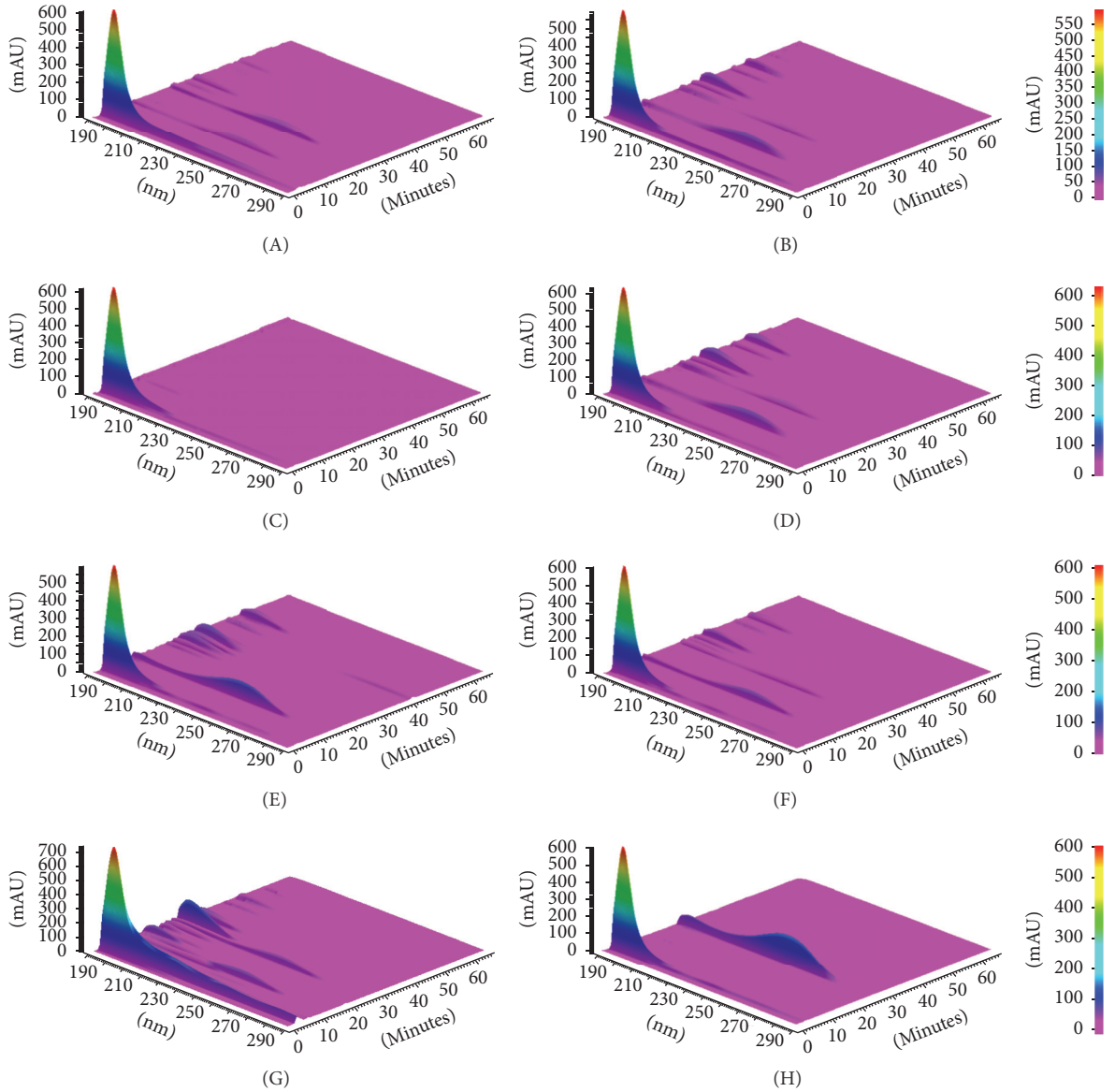


Figure 2: Chromatograms of *Boswellia serrata* extracts (BS) after HPLC-DAD analysis. BS (A)–(F) extracts were diluted 1 : 400 in methanol, whereas, BS (G) extract was diluted 1 : 20 in methanol. The chromatogram of the AKBA analytical standard is also reported (H). The absorbance (mAU) is reported on the *y*-axis, wavelength (nm) on *x*-axis, and the retention time (minutes) on the *z*-axis.

Table 2: In vitro radical scavenging activity and polyphenolic content of different *Boswellia* extracts.

Boswellia serrata extracts	Polyphenols mg GAE/g	DPPH		ABTS		FRAP
		TEAC[a] μmol TE/g	IC_{50}[b] μg/ml	TEAC μmol TE/g	IC_{50} μg/ml	TEAC μmol TE/g
A	7.68 ± 0.9	31.8 ± 0.7	340.2 ± 3.5	151.8 ± 10.6	79.26 ± 1.8	66.89 ± 3.5
B	1.43 ± 0.5	4.48 ± 0.08	2416 ± 12.5	37.54 ± 2.5	320.30 ± 4.5	ND
C	0.56 ± 0.2	3.83 ± 0.08	2823 ± 27.5	1.92 ± 0.1	6250 ± 17.5	ND
D	1.09 ± 0.4	6.29 ± 0.12	1720 ± 13.8	27.8 ± 1.9	431.62 ± 6.5	ND
E	1.09 ± 0.3	5.41 ± 0.11	1998 ± 17.5	18.7 ± 1.2	641.75 ± 9.5	ND
F	0.96 ± 0.3	5.20 ± 0.15	2080 ± 22.5	20.6 ± 21.4	581.94 ± 8.5	ND
G	0.11 ± 0.05	1.85 ± 0.03	5820 ± 32.5	1.76 ± 0.1	6800 ± 22.5	ND
Positive control						
Trolox			10.85 ± 0.2		3.01 ± 0.2	

[a]TEAC = Trolox equivalent (TE) antioxidant concentration. [b]IC_{50} = The concentration of compound that affords a 50% reduction in the assay; GAE = gallic acid equivalent. ND = not detectable.

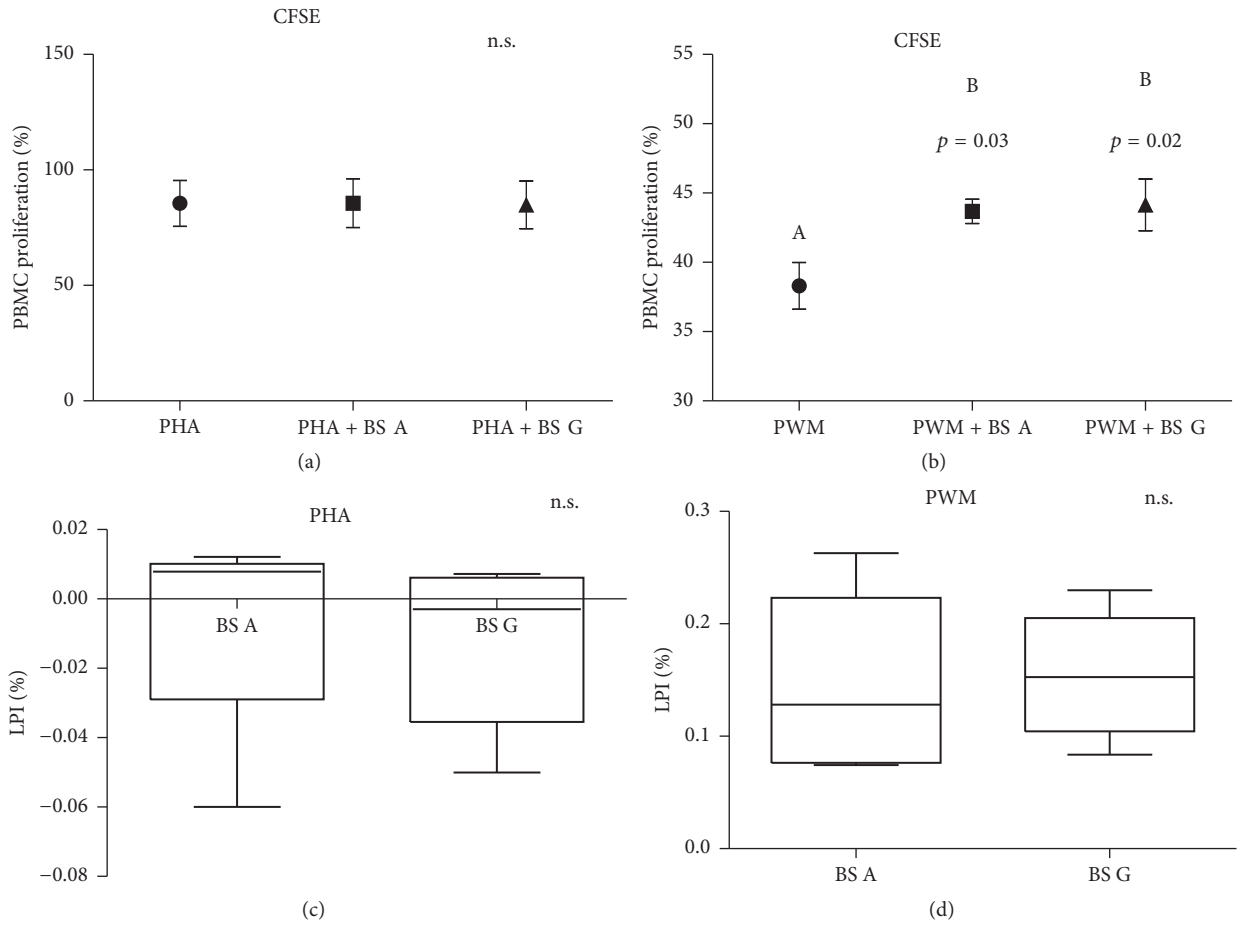


Figure 3: *Boswellia serrata* (BS) extract (A or G) effects on lymphocyte proliferation assay. Data are shown as mean ± SEM of seven independent experiments. PBMCs were cultured with phytohemagglutinin (PHA; graphics (a) and (c)) or pokeweed mitogens (PWM; graphics (b) and (d)) and stained with carboxyfluorescein diacetate succinimidyl ester cell tracer (CFSE). The lymphocyte proliferation index (LPI) was calculated as reported in the text. ^{A,B}Different letters for $p < 0.05$. n.s. = not significant.

However, when cells were not PHA pulsed, an increase of FOXP3$^+$ cells was observed in PBMCs cultured with the BS extracts. In particular, a tendency towards a higher number of regulatory cells was observed for extract A ($p = 0.079$), whereas extract G led to a significant increase of FOXP3$^+$ cells ($p = 0.045$; Figure 4(a)).

Furthermore, higher number of Th17$^+$ cells, although not significant, was again observed when PBMCs were cultured with the extract G (Figure 2(C)).

4. Discussion

The therapeutical efficacy of BS extracts has been extensively investigated in arthritis, asthma, diabetes mellitus, colitis, and cancer [26, 27] in light of their antioxidant and anti-inflammatory activities [28]. Indeed, all these diseases share a persistent dysregulation of redox status that contributes to the intensity and duration of the inflammatory response and therefore to the induction and perpetuation of chronic inflammation.

It is noteworthy that the phytochemical content of *B. serrata* oleogum resin is dependent on both the botanical origin and the geographical origin [29]. Usually it consists of 30–60% triterpenes (such as α- and β-boswellic acids, lupeolic acid), 5–10% essential oils, and 20–35% polysaccharides [30]. According to Singh et al. [31], in aqueous and ethanolic extracts of *B. serrata*, it is possible to recognize alkaloids, carbohydrates, phytosterols, terpenoids, phenolic compounds, flavonoids, and tannins. However, other authors found also glycosides, proteins, and saponins [32].

Furthermore, the wide variations of pharmacologically active molecules in commercial BS formulations could significantly affect the final product [33].

The first aim of this study was to perform a comparative analysis on the composition of different dry and aqueous extracts of *B. serrata* gum resin as a tool for the evaluation of the quality of the extracts.

Combination of TLC and HPLC analyses can be considered as a multidimensional analytical approach combining fast qualitative screening with an accurate and precise quantification of specific compounds. We decided to quantify AKBA because the boswellic acid, characteristic and unique to *Boswellia* genus, is considered the most effective, at least in *in vitro* studies. When analyzed at 260 nm, four of the

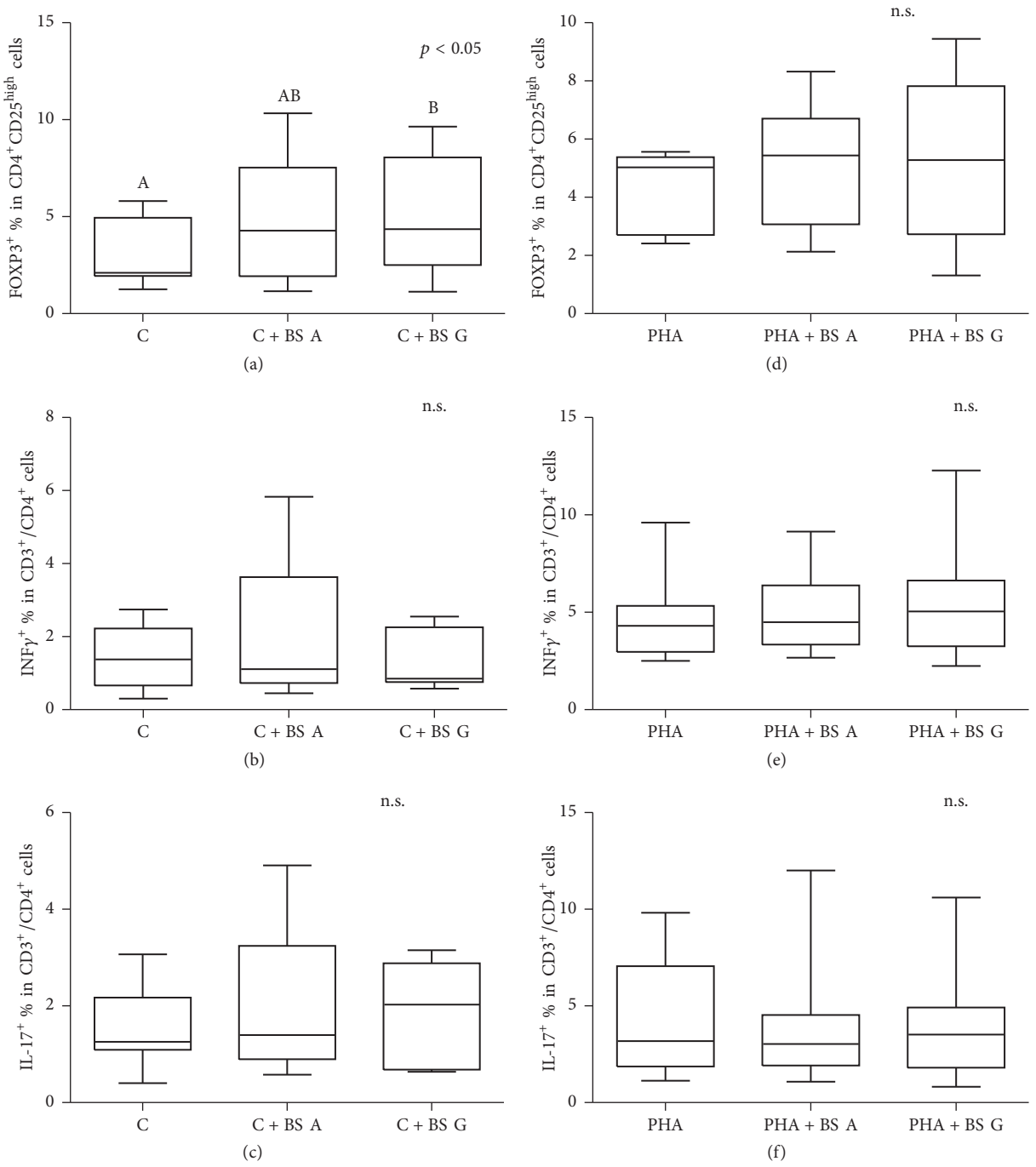


Figure 4: *Boswellia serrata* (BS) extract (A or G) effects on Treg ($CD4^+CD25^+FOXP3^+$ cells), Th1 lymphocyte ($INF\gamma^+$ cells), and Th17 cell ($IL\text{-}17^+$) responses. Data are shown as mean ± SEM of seven independent experiments. PBMCs were cultured in absence (controls, C; graphics (a), (b), and (c)) or presence of mitogen (phytohemagglutinin, PHA; graphics (d), (e), and (f)) and with/without BS extracts. ^{A,B}Different letters for $p < 0.05$. n.s. = not significant.

seven samples presented comparable profiles, characterized by the presence of two main peaks the second of which corresponds to AKBA. The other components of BS extracts, lacking the keto moiety, were visualized only at a less specific wavelength (210 nm) as already reported [34]. The AKBA concentrations detected in samples A, B, D, and F are similar to those reported by other authors [30, 35], while those found in extracts C and G were 10 or 100 times lower, respectively. It can be hypothesized that extract C belongs to *Boswellia* species other than *B. serrata*, due to a wrong botanical identification by local producers. Indeed, it has already been reported that the elution profile of *Boswellia frereana* gum resin lacks KBA and AKBA peaks [34], while *Boswellia sacra* gum resin contains much lower amounts of KBA and less AKBA than *B. serrata* [34, 36]. Concerning extract G, this aqueous extract showed a lower content of AKBA, but it

was enriched by other components of the phytocomplex, as demonstrated by the additional peaks obtained in the elution profile at 210 nm. The presence of these compounds is probably related to the particular and innovative extraction method [16].

The composition of BS extract E is challenging, due to the discrepant results in TLC and HPLC analysis. The spot at Rf of 0.26, a putative AKBA component, was not confirmed by a corresponding peak in the HPLC chromatogram at 260 nm. Other components of the phytocomplexes containing a keto moiety should have contributed to this spot and further analyses are needed to identify these molecules.

The antioxidative potential and radical scavenging activity of aqueous and ethanolic extracts of *B. serrata* are significantly correlated to their total phenolic and flavonoid content [31].

According to Kohoude et al. [37], the amount of phenolics in *Boswellia* genus (315 g/kg) is comparable to reference extracts rich in phenolic compounds. Despite what is reported in literature, the BS samples investigated here were all characterized by either relatively low antioxidant properties or total phenolic content. Indeed, the latter ranged from 0.11 ± 0.05 to 7.68 ± 0.9 mg GAE/g versus values of 28.46–12.73 mg GAE/g obtained by other authors in aqueous and ethanolic extracts, respectively [31].

According to the extraction procedure, the antioxidant activity increased with the polarity of the solvent. Other authors reported that the essential oil of *Boswellia dalzielii* was characterized by low antioxidant activity [36] and that this was due to the extraction method adopted (e.g., low polarity of the solvent) that determined the absence of phenolics, especially flavonoids.

In the present study, the extraction methods adopted by manufacturers or even the preservative systems used could be responsible for the low level of total phenolic and flavonoids compounds, which are contained mainly in the volatile essential oil component of the oleogum resin.

Furthermore, although theoretically the aqueous extract (extract G) should have characteristics closest to the natural product being obtained by an enzymatic hydrolysis that maintains the intact phytocomplexes [16], we observed that it was the one with the lowest antioxidant properties and total phenolic contents.

However, these results are at least partially in line with those of other authors reporting that the wild habitat samples, with a completely different profile as compared to the market samples, were those lacking antioxidant activity [28].

Nevertheless, the BS extracts of the present study were able to significantly modulate some immune responses investigated independently of the in vitro antioxidant activities. As reported in Figure 3(b), when cells were stimulated by PWM, a mitogen that stimulates B lymphocytes in the presence of T-cells, the PBMC proliferation was significantly increased ($p < 0.05$) by the addition of the BS extracts (0.1 μg/mL for both extracts A and G) and the LPI did not change between the two BS extracts (Figure 3(d)).

Conversely, when cells were activated by PHA, neither the PBMC proliferation (mainly, T-cells$^+$) nor the LPI were affected by the BS extracts (Figures 3(a) and 3(c)).

It has been previously reported that the BS could produce opposing effects on immune responses in vivo or in vitro. Potentially low concentrations of BSs increase stimulated proliferation of lymphocytes whereas higher concentrations are even inhibitory [14].

Sharma et al. [38] reported that a mixture of various boswellic acids in the range of 1.95–125 μg/mL inhibited mice splenocytes stimulated with lipopolysaccharides (LPS), PHA, alloantigen, and concanavalin A (ConA), in a concentration-dependent manner. Indeed, a significant inhibition of splenocytes to mitogens and alloantigens was observed starting from concentrations greater than 3.90 μg/mL.

On the other hand, Gayathri et al. [15] observed that 30 μg/mL of a crude methanolic BS extract is able to inhibit almost 80% of human lymphocyte proliferation. These data are in striking contrast with the observations of other authors [39] who tested the effect of 1 mg/mL of BS total alcoholic extract, gum, or volatile oil on human lymphocyte proliferation and observed no inhibition of cells stimulated with either PHA or Con A.

Besides the different lymphocyte proliferation assays applied, the BS concentrations used in the present study were 10 to 1000 times lower than those adopted in the cited articles and, at these doses, we obtained an effect on the lymphocyte proliferation only when cells were stimulated by PWM (B cells$^+$).

However, in mice treated with orally administered boswellic acids, the secondary antibody titres were appreciably enhanced at the lowest tested doses (25 mg/kg body weight versus 100–200 mg/kg) [38].

The dose of BS extracts we adopted was probably low enough to induce an effect on B cells$^+$ (activated by PWM), but too low to induce an inhibition of T lymphocyte proliferation, as reported by other authors [14, 39].

In the maintenance of T-cell balance a pivotal role is attributed to T-helper cells and regulatory T-cells [40, 41]. T-helper cells are defined as Th1-, Th2-, or Th17-cells and are characterized by differential expression of certain cytokines [42]. Th1-cells have the capacity to express the key cytokine interferon-γ (IFN-γ), whereas Th17-cells, a more recently described T-helper cell subset, evolutionally and functionally divergent from Th1 and Th2 cell subsets, are characterized by their ability to produce interleukin-17A (IL-17A) [43].

Regulatory T-cells (Tregs) suppress effector T-cells and, in humans, can be characterized by a $CD4^+CD25^{high}FoxP3^+$ phenotype [41].

In our study, we observed that when PBMCs from healthy controls were not activated by PHA mitogen, the presence of BS extract G in the culture medium determined a significant increase of Tregs (Figure 4(a)). Furthermore, the increased number of Treg cells in BS G treated samples was accompanied by a higher number, although not significant, of Th17$^+$ cells. Conversely, the BS extracts did not influence the number of Th1$^+$ cells (INFγ^+). When PBMCs were pulsed by PHA, no additional effect could be seen following the BS extract addition.

The recent evidence of a developmental plasticity between Treg and Th17 cells prompts the investigation of intermediate phenotypes that result from their reciprocal conversion

according to the surrounding microenvironment [44]. The presented results show a possible role for BS extracts in such a fine balance between these two cell subsets.

Furthermore, it is important to note that, at least in mice, Th17 lymphocytes can also function as B-cell helpers [45], mediating B-cell differentiation and antibody class switch recombination. The results of our lymphocyte proliferation assay showed that the BS extracts exerted a significant stimulatory effect on B+ cell proliferation, possibly mediated by an enhanced number of $Th17^+$ cells.

Many authors have demonstrated that *B. serrata* extracts turn out to be effective in the treatment of diseases such as inflammatory bowel disease and osteoarthritis in which inflammation and/or oxidative stress exert an important pathogenic role [2, 13, 30, 46].

However, BS extracts also exerted beneficial effects in some autoimmune diseases, such as rheumatoid arthritis [1], where chronic inflammation and an aberrant autoimmune response are hallmarks of the disease [47].

This *ex vivo* study provides evidence that *B. serrata* extracts, besides their reported capacity in dampening the inflammatory response together with counteracting the oxidative stress, were able to influence the regulatory and effector T-cell compartments.

In order to draw conclusions, it will be necessary to deepen the experiment on a wider case study. However, these preliminary results suggest that frankincense should be further investigated for its promising ability to interfere, possibly also through such regulatory mechanism, on immune dysregulation typical of various immune disorders, but attention should be paid to the quality of the commercial extracts which can show wide variations in their chemical composition.

Conflicts of Interest

Authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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Research Article

Evaluation of Anti-Inflammatory Properties of Isoorientin Isolated from Tubers of *Pueraria tuberosa*

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Inflammation is the major causative factor of different diseases such as cardiovascular disease, diabetes, obesity, osteoporosis, rheumatoid arthritis, inflammatory bowel disease, and cancer. Anti-inflammatory drugs are often the first step of treatment in many of these diseases. The present study is aimed at evaluating the anti-inflammatory properties of isoorientin, a selective cyclooxygenase-2 (COX-2) inhibitor isolated from the tubers of *Pueraria tuberosa*, in vitro on mouse macrophage cell line (RAW 264.7) and in vivo on mouse paw edema and air pouch models of inflammation. Isoorientin reduced inflammation in RAW 264.7 cell line in vitro and carrageenan induced inflammatory animal model systems in vivo. Cellular infiltration into pouch tissue was reduced in isoorientin treated mice compared to carrageenan treated mice. Isoorientin treated RAW 264.7 cells and animals showed reduced expression of inflammatory proteins like COX-2, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), 5-lipoxygenase (5-LOX), and interleukin 1-β (IL-1-β) both in vitro and in vivo. The antioxidant enzyme levels of catalase and GST were markedly increased in isoorientin treated mice compared to carrageenan treated mice. These results suggest that isoorientin, a selective inhibitor of COX-2, not only exerts anti-inflammatory effects in LPS induced RAW cells and carrageenan induced inflammatory model systems but also exhibits potent antioxidant properties.

1. Introduction

Inflammation is a cellular, immune, and metabolic response to injury/infection. It is a normal protective vascular connective tissue response to eliminate the cause of injury and clean up the dead and dying cells but when it occurs in uncontrolled or inappropriate manner it results in pathogenesis of several disorders which include cardiovascular, respiratory, neurological, and many lifestyle diseases. Inflammation is a complex interplay of cellular and particulate mediators, which include chemokines, plasma enzymes, lipids, and cytokines. Among these the lipid mediators such as eicosanoids, the oxygenated metabolites of arachidonic acid formed via the cyclooxygenase (COX) and lipoxygenase (LOX) pathways, play a predominant role in mediating the inflammatory disorders. The anti-inflammatory drugs are being targeted against COX pathway. These include the conventional nonsteroidal anti-inflammatory drugs (NSAIDs) that target both COX-1 and COX-2 and the selective COX-2 inhibitors (COXIBs). Although these drugs are effective in controlling signs of inflammation, number of adverse effects encountered is the biggest limitation to their use [1, 2]. These include the gastric

side effects of traditional NSAIDs and the cardiac side effects of COXIBs.

Traditional medicinal practices have been known for millennia for the treatment of various ailments [3, 4]. Over three-quarters of world population are relying mainly on plants and plant extracts for health care [3–5]. Medicinal plants produce bioactive compounds used mainly for medicinal purposes. Recently it was shown that tuberostemonine N, isolated from *Stemona tuberosa*, suppresses cigarette smoke induced subacute lung inflammation in mice [6]. Similarly it was shown that natural products like (7R, 8S)-9-acetyl-dehydrodiconiferyl alcohol (ADDA), isolated from *Clematis armandii* stems [7] and mitraphylline from *Uncaria tomentosa* [8], exert anti-inflammatory properties. The present study is aimed at determining the anti-inflammatory effects of isoorientin isolated from the tubers of *Pueraria tuberosa*. *P. tuberosa* herb is used as a cardiotonic, galactagogue, and diuretic, as well as for fertility control in folk medicine. The tuber extracts are known to have antioxidant, hypoglycemic, hypolipidemic, anti-inflammatory, and in vivo immunomodulatory activities [9–12]. Earlier, we have shown that isoorientin selectively inhibits COX-2 suggesting its potential as a promising anti-inflammatory drug candidate [13]. Present study evaluates the anti-inflammatory potential of isoorientin in vitro on mouse macrophage cell line, RAW 264.7, and on paw edema and air pouch models of inflammation in vivo.

2. Materials and Methods

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), trypsin-EDTA, Tris, ethylenediaminetetraacetic acid (EDTA), diethyldithiocarbamate (DDC), Tween-20, hematin, glycerol, phenol, and ammonium sulphate were purchased from Sigma Chemical Co. (St. Louis, MO). Celecoxib was a generous gift from Unichem Laboratories (Mumbai, India). Carrageenan was purchased from Sigma-Aldrich (St. Louis, USA). Polyclonal antibodies to COX-2, IL-6, 5-LOX, IL-1β, and TNF-α were purchased from Santa Cruz Biotechnology (California, USA). iNOS antibody was from Thermo Fisher Scientific Inc. All other chemicals and solvents were of analytical grade and purchased from authorized standard companies. Isoorientin was isolated from the methanolic extracts of tubers of *P. tuberosa* [13].

2.1. Cell Culture. RAW 264.7 (murine macrophages) cell line was obtained from National Centre for Cell Science (NCCS), Pune. The cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C. Medium for all the cell lines was DMEM supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine.

2.2. Effect of Isoorientin on Cell Viability. RAW 264.7 cells viability in the presence and absence of isoorientin was assessed using MTT assay. Cells were treated with different concentrations of isoorientin (25 nM to 100 μM) for 16 h and then the cell viability was assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (0.5%) to purple formazan. Cells were then incubated with MTT (0.5%) for 4 h at 37°C. The medium was then removed by aspiration and formazan crystals were dissolved in DMSO. The extent of the reduction of MTT was quantified by measurement of absorbance at 570 nm using microtiter plate reader.

2.3. SDS-PAGE and Western Blotting. An equal quantity of cytosolic/nuclear proteins from each treatment (75 μg of total protein/lane) was resolved on 8–12% SDS-PAGE gels and then transferred onto nitrocellulose membranes. Membranes were stained with 0.5% Ponceau in 1% acetic acid to confirm equal loading. The membranes were blocked with 5% w/v nonfat dry milk and then incubated with the primary antibodies in 10 mL of antibody-diluted buffer (Tris-buffered saline and 0.05% Tween-20 with 5% milk) with gentle shaking at 4°C for 8–12 h and then incubated with respective conjugated secondary antibodies. Signals were detected using Western blot detection reagents.

2.4. Preparation of Cytoplasmic and Nuclear Extracts. RAW 264.7 cells were cultured in 6-well plates (4×10^6 cells/well) with or without LPS (1 μg/mL) and in the presence or absence of isoorientin (0–25 μM). The cytoplasmic and nuclear protein extracts were prepared for measuring the protein levels by Western blotting and enzyme immunoassay (EIA). Briefly, after culture the cells were collected and washed twice with cold PBS and lysed in 400 μL of cold buffer A (HEPES 10 mmol/L pH 7.9, KCl 10 mmol/L, 1 mM EDTA, phenylmethanesulphonylfluoride (PMSF) 1 mmol/L, 1 mM EGTA, dithiothreitol (DTT) 1 mmol/L, aprotinin 1 mg/L, leupeptin 1 mg/L, and pepstatin A 1 mg/L). After 15 min incubation on ice, 0.1% NP-40 was added to the homogenates and the tubes were vigorously rocked for 1 min. Then the homogenates were centrifuged (20,800 ×g, 5 min) in a microcentrifuge at 4°C. The supernatant fluid (cytoplasmic extracts) was collected and stored in aliquots at −70°C. The nuclear pellets were washed once with cold buffer A, then suspended in 50 μL of cold buffer B (HEPES 20 mmol/L, pH 7.9, NaCl 420 mmol/L, edetic acid 0.1 mmol/L, egtazic acid 0.1 mmol/L, PMSF 1 mmol/L, DTT 1 mmol/L, aprotinin 1 mg/L, leupeptin 1 mg/L, and pepstatin A 1 mg/L), and vigorously rocked at maximum speed at 4°C for 30 min. The solution was clarified by centrifugation at 20,800 ×g for 5 min, and the supernatant fluid (nuclear extract) was stored in aliquots at −70°C. The protein concentration was determined according to the Bradford method [14].

2.5. Experimental Animals. Adult Balb/c male mice weighing 20–25 g were used for all the experiments in the present study. They were fed with a standard chow pellet diet, had free access to water, and were maintained on a 12 : 12-h light-dark cycles. All procedures in this study were approved by the Animal Ethical Committee of the National Institute of Animal Biotechnology.

2.6. Paw Edema Model. The study was performed on carrageenan induced paw edema in Balb/c mice with swelling measured using electronic vernier calipers. Paw edema was induced in Balb/c mice by subcutaneous injection of carrageenan (0.1 mL of 1% solution w/v in 0.9% saline) into subplantar region of the left hind paw and these animals were

divided into 5 different groups (6 mice in each group) as described below in administration of isoorientin. Edema was calculated as the average difference of paw thickness (mm) in treated groups compared with that in control groups (DMSO treated).

2.7. Air Pouch Model of Inflammation. Carrageenan treated mice air pouch model of inflammation was developed as described previously [15]. Air cavities were produced by subcutaneous injections of 5 mL of sterile air into the intracapsular area on the dorsal side of the animal. An additional 3 mL of air was injected into the cavity every three days. Seven days after the initial air injection, 0.5 mL of 1.5% (w/v) solution of carrageenan dissolved in saline was injected directly into the pouch to produce an inflammatory response. For the time course studies, animals were sacrificed by cervical dislocation at various time points after the injection. Pouch tissue was carefully dissected and cut open to aspirate the inflammatory exudates into graduated tubes. The pouch lining was separated from the muscle and dissected out and rinsed in saline before processing further. Cell population in the pouch cavity was measured by gavage of about 2 mL of saline into the pouch repeatedly. This procedure ensures the complete recovery of cells from the pouch. For cell counting the collected fluid was centrifuged and the cell pellet was washed in RPMI medium twice to remove the debris and dissolved in saline and then counted on hemocytometer.

2.8. Administration of Isoorientin. In the case of paw edema model the isoorientin or celecoxib was given intraperitoneally and carrageenan was injected into the paw directly one hour later. In air pouch model all the treatments were given along with carrageenan directly into the pouch cavity. Isoorientin was injected three hours earlier than injection of carrageenan into the pouch cavity. Isoorientin and celecoxib were administered into the mice air pouch. The stock solutions of isoorientin (100 mg/mL) and celecoxib (100 mg/mL) were prepared in DMSO and further dilutions were made at the time of treatments. Animals were divided, into 5 different groups as follows: control (DMSO treated); carrageenan (0.5 mL of 1.5% (w/v) carrageenan in saline) treated; carrageenan + celecoxib (20 mg/kg body weight) treated; carrageenan + isoorientin (10 mg/Kg body weight) treated; carrageenan + isoorientin (20 mg/Kg body weight) treated.

2.9. Histology of Air Pouch Tissue. Air pouch tissues from control and experimental animals were rinsed in PBS and fixed in Bouin's fixative (70% saturated picric acid, 25% formaldehyde, and 5% glacial acetic acid) overnight followed by thorough washing with distilled water. Tissues were then dehydrated sequentially in 70%, 80%, and 90% alcohol and finally in absolute alcohol for 10 min each. After dehydration, the tissue was processed in alcohol and benzene (3 : 1 for 10 min, 1 : 1 for 10 min, benzene and paraffin (1 : 1) for 10 min) to embed in paraffin wax. The tissue was placed in molten paraffin for 2-3 h to allow infiltration of paraffin into the tissue and then allowed to harden. Thin sections (10 μm) were taken on Leitz microtome and mounted on polylysine-coated slides. Sections were deparaffinised by incubating in xylene for 10 minutes and rehydrated by sequential incubations in 90, 80, and 70% alcohol for 10 minutes each. The tissue sections were observed under light microscope at 400x magnification and photographs were taken.

2.10. SDS-PAGE and Western Blotting. Pouch tissue homogenate was prepared by homogenizing the pouch lining tissue in 100 mM Tris-HCl (pH 8.0) buffer containing 0.3 M mannitol, 1 mM EGTA, 1 mM EDTA, 4 mM K_2HPO_4, 1 mM DTT, 1 mM sodium orthovanadate, 0.1% SDS, 2 mM PMSF, and 40 μL/mL of complete protease inhibitor solution. The homogenate was centrifuged for 30 min at 10,000 rpm at 4°C and the resultant supernatant was used for SDS-PAGE and Western blot analysis. Protein content in the supernatant was measured by Lowry method [16]. SDS-PAGE and Western blot analyses for the detection of COX- 2, iNOS, IL-1β, TNF-α 5-LOX, and β-actin in the air pouch tissue homogenate were performed by the procedure mentioned earlier.

2.11. Catalase Assay. Catalase activity was measured using standard protocol described earlier by Aebi [17]. The activity of catalase present in the samples was determined by the decomposition of H_2O_2, which can be monitored at 240 nm. 30 mM H_2O_2 was mixed in 50 mM phosphate buffer. The assay mixture contained 10 μL of sample and 1 mL of 30 mM H_2O_2. The change in the optical density (OD) was monitored for 3 min at 30 sec intervals. The activity of catalase in the diluted sample was calculated using the first-order reaction:

$$K_{30} = \left(\frac{2.303}{30}\right) * \log\left(\frac{A_1}{A_2}\right). \quad (1)$$

The activity in the sample was expressed as K_{30}/mg protein.

2.12. Estimation of Glutathione-S-Transferase (GST) Activity. Estimation of GST was carried out using the methodology described earlier by Jakoby [18]. GST catalyses the formation of a conjugate between GSH and a variety of substrates. In this method, 1-chloro-2,4-dinitrobenzene was used as the substrate. The formation of GSH-CDNB catalysed by GST was monitored at 340 nm and the amount of the conjugate formed is a measure of the enzyme activity. The reaction mixture contained 924 μL of phosphate buffer pH, 33.3 μL of CDNB solution (10 mM), and 10 μL of the sample. The reaction was initiated by adding 33.3 μL of GSH solution (10 mM) to the reaction mixture and the change in the OD was monitored at 340 nm for 5 min. The activity of the enzyme in the sample was calculated using the following formula:

$$\text{GST activity} = \frac{\text{Abs difference } (\Delta D/\text{min}) \times 1 \times 100 \ \text{(dilution factor)}}{7.6 \times 5 \times \text{protein in mg}}. \quad (2)$$

Activity of GST was expressed as μmoles CDNB-GSH conjugate formed/min × mg protein.

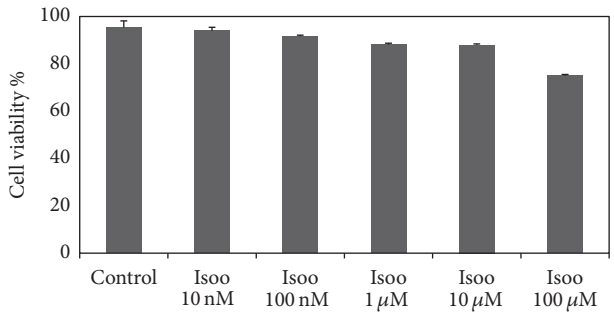

Figure 1: Effect of isoorientin on RAW 264.7 cells viability. The cells were incubated with or without isoorientin along with LPS (1 μg/mL) for 16 hrs and then the cell viability as measured by MTT assay was determined. The percent cell growth was calculated in comparison with untreated control cells. Data are mean ± SEM of three independent experiments ($N = 3$).

3. Statistical Analysis

The number of animals used in each treatment group is six ($n = 6$). Data were expressed as mean ± standard error. Correlations between the various parameters were analyzed using regression analysis. p value was determined by the Student's t-test. p value of less than 0.05 was considered as a significant difference.

4. Results

4.1. Effect of Isoorientin on RAW 264.7 Cell Viability. To study the cytotoxicity of isoorientin, RAW 264.7 cells were incubated with different concentrations of isoorientin (10 nM to 100 μM) along with LPS (1 μg/mL) for 16 h. No significant effect of isoorientin was observed on the growth of RAW cells, suggesting no cytotoxicity of isoorientin up to 100 μM (Figure 1).

4.2. In Vitro Effect of Isoorientin on the Expression of COX-2, iNOS, 5-LOX, TNF-α, and IL1-β. Expression levels of inflammatory proteins like COX-2, iNOS, 5-LOX, TNF-α, and IL1-β were studied in RAW 264.7 cells pretreated with isoorientin and then challenged with or without LPS. RAW 264.7 cells were pretreated with 1 μM, 5 μM, 10 μM, and 25 μM concentrations of isoorientin and after 3-4 h of preincubation cells were induced with 1 μg/mL LPS. Control cells did not receive any LPS. Celecoxib was used as the standard anti-inflammatory drug. The results revealed increased expression of all the inflammatory markers studied in cells treated with LPS. However, the expression levels were decreased in isoorientin treated cells in a dose dependent manner (Figure 2) and isoorientin showed decreased expression of inflammatory proteins better than standard drug.

4.3. Effect of Isoorientin on LPS Induced NF-κB Activation. As NF-κB is required for the activation of inflammatory proteins like COX-2 and iNOS, Western blot was performed to check whether isoorientin suppresses LPS induced NF-κB activation. Since p65 is one of the major components of NF-κB, we examined the translocation of NF-κB from cytosol to nucleus by Western blotting. Negligible levels of p65 were detected in control nuclei, but treatment with LPS alone for 1 h resulted in marked increase of p65 levels in the nucleus. Pretreatment with isoorientin or celecoxib inhibited LPS induced nuclear translocation of p65 subunit of NF-κB in a concentration dependent manner (Figure 3).

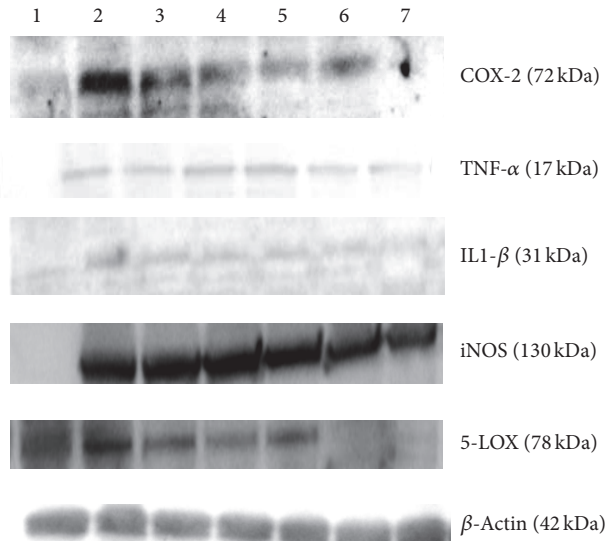

Figure 2: In vitro effect of different isoorientin concentrations on the expression of COX-2, TNF-α, IL-1-β, iNOS, and 5-LOX, by Western blot. (lane 1) Control cells, (lane 2) LPS alone (lane 3), LPS + celecoxib, (lane 4) LPS + Isoorientin 1 μM, (lane 5) LPS + Isoorientin 5 μM, (lane 6) LPS + Isoorientin 10 μM, and (lane 7) LPS + Isoorientin 25 μM.

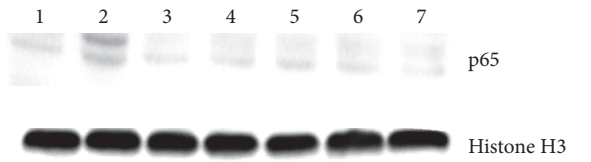

Figure 3: Effect of isoorientin on translocation of NF-κB in LPS stimulated RAW 264.7 cells. Nuclear extracts were prepared from controls or pretreated with different concentrations of isoorientin for 1 h and then induced with LPS for 1 h and analyzed for NF-κB translocation.

4.4. Isoorientin Inhibits Paw Swelling in a Murine Paw Edema Model. Paw edema was induced in mice using carrageenan, and mice were treated with isoorientin. Celecoxib was used as a positive control. Intraplantar injection of carrageenan caused an increase in the thickness of mouse paw. This paw edema peaked at 3 hour after carrageenan induction, with a thickness of 1.48 ± 0.04 mm and 0.73 ± 0.03 mm in celecoxib group. Animals treated with isoorientin at 10 mg/kg and 20 mg/kg body weight had a statistically significant reduction in paw edema, with a mean peak thickness of 1.19 ± 0.05 mm and 1.08 ± 0.04 mm, respectively. This indicated that isoorientin significantly attenuated paw edema compared with the control group (Figures 4(a) and 4(b)).

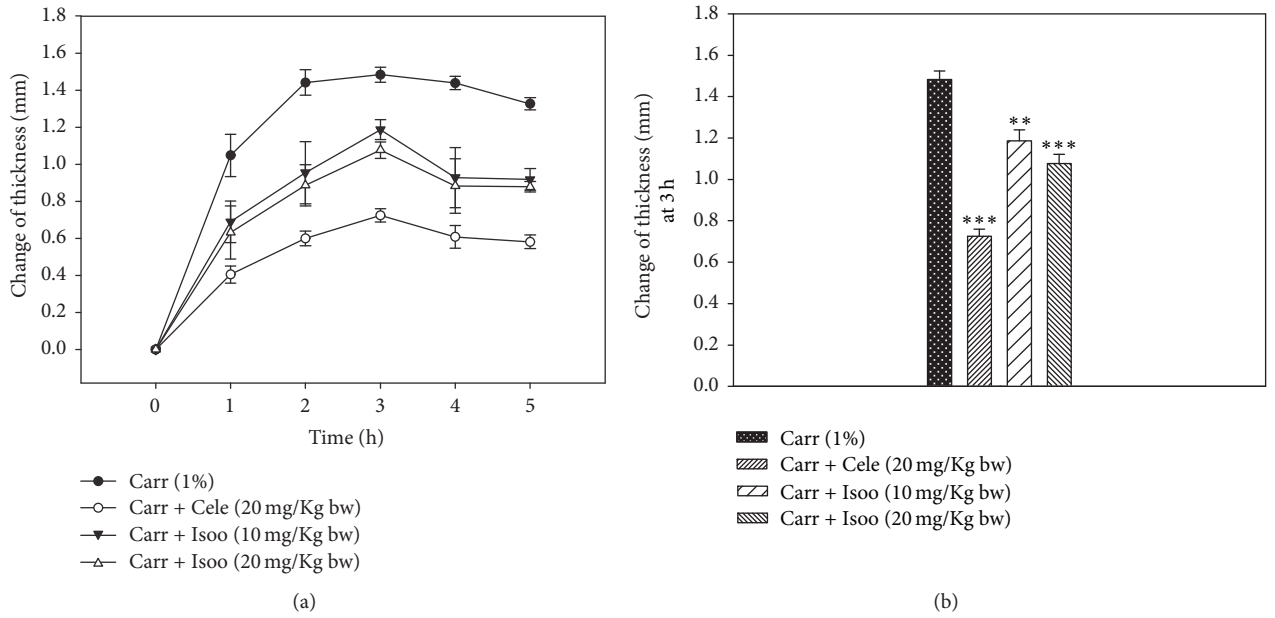

FIGURE 4: Effect of isoorientin on carrageenan induced paw edema in BALB/c mice. Animals were injected with isoorientin (10 and 20 mg/Kg body weight), celecoxib (20 mg/Kg body weight), or an equal volume of the vehicle (50 μL, 0.2% DMSO) intraperitoneally. One hour later, paw inflammation was induced by injecting 25 μL of 1% solution of carrageenan in 0.9% saline subcutaneously into the plantar region of the left hind paw. (a) The thickness of the paw was measured in the dorsal plantar axis at the metatarsal level by digital calliper at the indicated times after carrageenan injection. (b) The thickness of the paw edema at 3 hour after induction was shown. All data are expressed from $n = 6$ as mean ± SD. $^{**}p < 0.01$; $^{***}p < 0.001$ compared with the control.

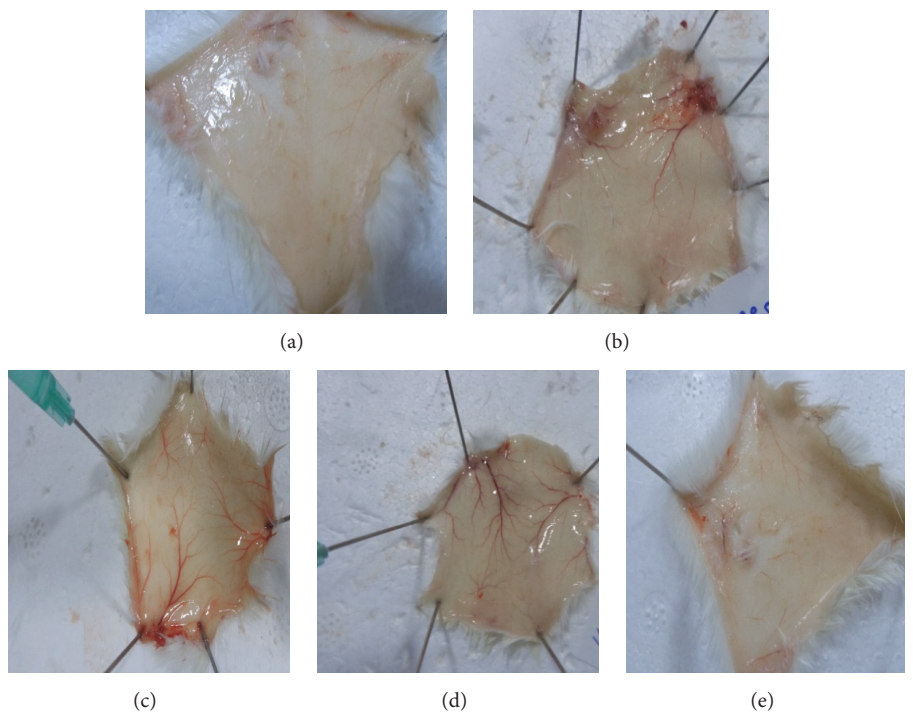

FIGURE 5: Effect of isoorientin on carrageenan induced blood vessel swelling in air pouch model in Balb/c mice. Photographs of air pouch tissue 24 hours after administration of (a) DMSO, (b) carrageenan, (c) carrageenan + celecoxib, (d) carrageenan + isoorientin 10 mg/kg body weight, and (e) carrageenan + isoorientin 20 mg/kg body weight.

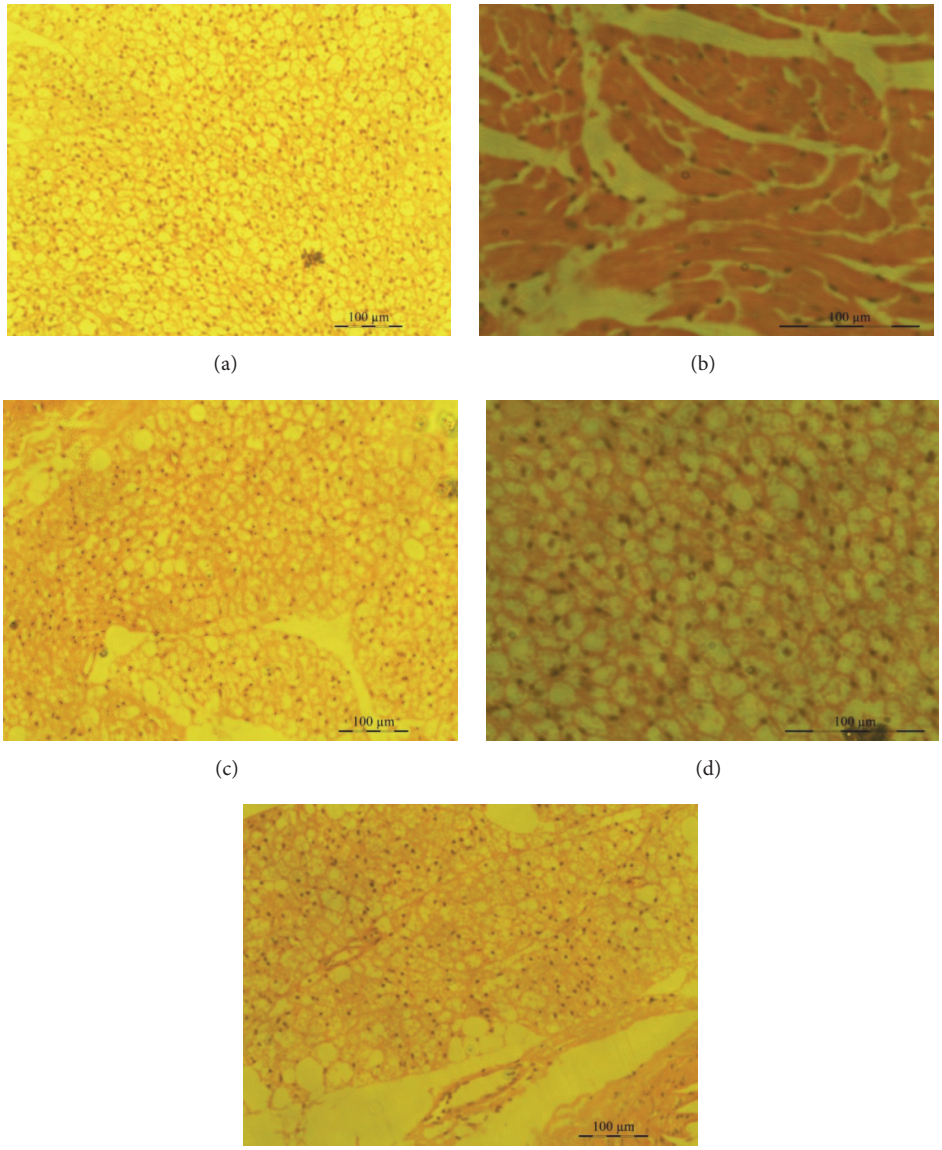

Figure 6: Histopathological observations of granulomatous tissue. Photomicrographs showing the histological sections of pouch tissue 24 hours after administration of (a) DMSO, (b) carrageenan, (c) carrageenan + celecoxib, (d) carrageenan + isoorientin 10 mg/Kg body weight, and (e) carrageenan + isoorientin 20 mg/Kg body weight.

4.5. Isoorientin Inhibits Inflammation in a Murine Air Pouch Model. Mice were treated with isoorientin at two different doses of 10 mg/kg body weight and 20 mg/kg body weight along with carrageenan. Control mice received DMSO alone. The classical symptoms of acute inflammation, redness and swelling, were clearly observed in the air pouch lining of carrageenan treated animal. The inflammatory reaction gradually progressed with time and reached a peak at 24 h after carrageenan treatment. In the isoorientin treated animals, the inflammatory reaction was less when compared to animals treated with carrageenan alone. Carrageenan treated pouch tissue showed increases in blood vessel size, while mice tissue of those treated with 10 mg/kg isoorientin showed smaller increases compared to carrageenan treated mice, which was almost completely abrogated in 20 mg/kg isoorientin treated mice (Figures 5 and 6). The number of cells infiltrated in to the air pouch were monitored in carrageenan alone or carrageenan along with isoorientin treated mice. The infiltration of cells increased with carrageenan treatment compared to control. However, this infiltration of cells was decreased when mice were treated with isoorientin in concentration dependent manner (Figure 7). These observations clearly demonstrate the anti-inflammatory effects of isoorientin in the air pouch model of the carrageenan treated animals.

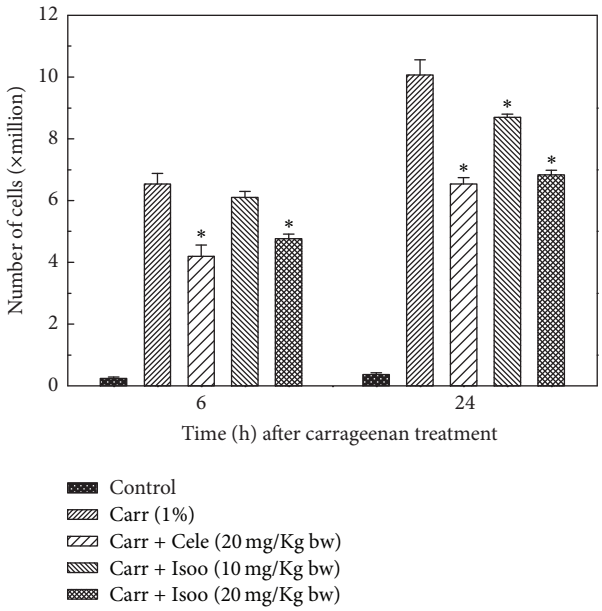

Figure 7: Effect of isoorientin on number of cells infiltrated into the air pouch of either carrageenan or carrageenan + isoorientin treated mice. Animals were sacrificed at various time points after treatments. The values were the mean ± SE of data obtained from 6 different animals. $^{*}P < 0.05$ compared to carrageenan treated animals.

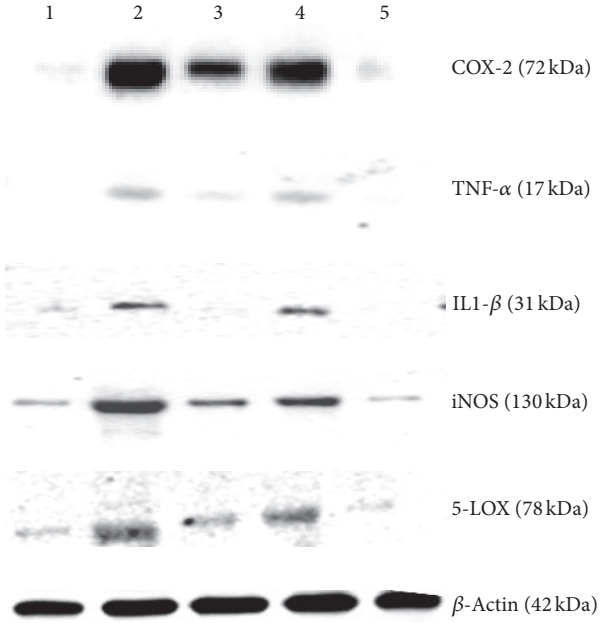

Figure 8: Effect of different isoorientin concentrations on the expression of COX-2, TNF-α, IL-1β, iNOS, and 5-LOX proteins in the mice air pouch tissue by Western blot. Treatment with (lane 1) DMSO, (lane 2) carrageenan, (lane 3) carrageenan + celecoxib, and (lane 4) carrageenan + isoorientin 10 mg/kg body weight and (lane 5) 20 mg/kg body weight.

4.6. Effect of Isoorientin on the Expression of Inflammatory Proteins. As isoorientin reduced inflammation in the mice air pouch model, further we examined the expression of inflammatory proteins COX-2, TNF-α, IL-1β, iNOS, and 5-LOX by Western blot (Figure 8). The expression of COX-2, TNF-α, IL-1β, iNOS, and 5-LOX was markedly upregulated in response to carrageenan; however, treatment of isoorientin decreased the expression of these proteins in a concentration dependent manner, and these effects are comparable to those observed with celecoxib treatment.

4.7. Effects of Isoorientin on Catalase and Glutathione S-Transferase. Catalase is critical in catalysing the decomposition of hydrogen peroxide to oxygen and water, thus protecting from oxidative damage due to reactive oxygen species (ROS). The effect of isoorientin on catalase activity was monitored in RAW 264.7 cells as well as tissues from the air pouch of carrageenan treated animals. The results indicate that, in RAW cells, catalase activity showed no significant effects with LPS treatment. However, the catalase activity increased in dose dependent manner in isoorientin treated cells (Figure 9(a)). In the tissues from the air pouch of carrageenan treated animals the catalase activity was similar as observed in control animals, whereas in the tissues of isoorientin treated mice catalase activity increased significantly (Figure 9(b)).

GSTs are a group of multigene multifunctional proteins involved in the detoxification of xenobiotics, including organic peroxides. GST activity was increased significantly in a dose dependent manner with isoorientin (Figure 10) in both LPS treated RAW cells and carrageenan induced inflammatory tissue.

5. Discussion

Natural products have been used as remedies since ancient times to combat several human disorders such as cardiovascular disease, cancer, and many inflammatory disorders [3, 5]. Drugs derived from natural products are making enormous contribution to drug discovery. Isoorientin is one such natural compound isolated from the tubers of *P. tuberosa*, which is a climbing, coiling, and trailing vine with tuberous roots. Tubers are used to reduce body dryness and for easy bowel movement. Isoorientin was isolated from methanolic extract of *P. tuberosa*. Earlier we have reported that isoorientin inhibited COX-2 with an IC_{50} value of 39 μM [13]. In this study, we have evaluated the anti-inflammatory effects of isoorientin in vitro on mouse macrophage cell line, RAW 264.7, and in vivo on paw edema and air pouch models of inflammation. Although inflammation is a normal response, when it occurs in an uncontrolled or inappropriate manner, excessive damage to host tissues and disease can ensue.

In the present study we showed that isoorientin does not affect the growth of RAW cells up to 100 μM conc. COX-2, iNOS, and 5-LOX serve as key mediators of inflammation. The agents that inhibit the expression of these proteins have therapeutic potential for inflammatory diseases. The expression levels of these proteins were increased on induction with LPS and were decreased on isoorientin treatment in dose

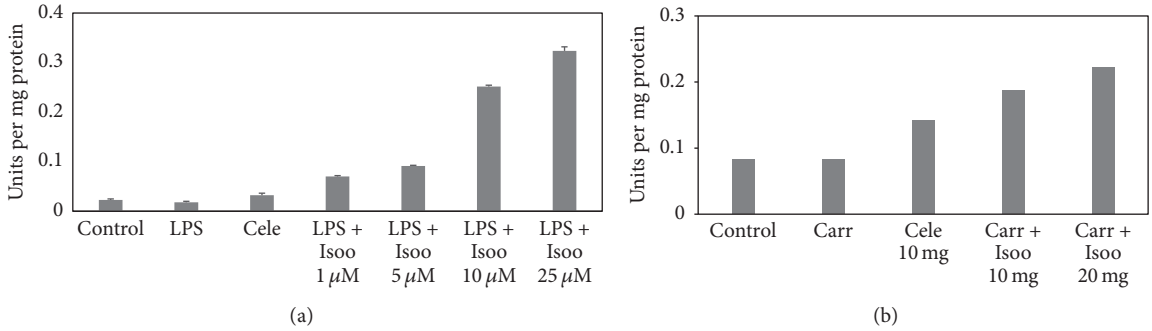

Figure 9: Effect of isoorientin on catalase activity in RAW 264.7 cells and air pouch tissue. Bar graph showing catalase activity in (a) RAW 264.7 cells, (bar 1) control cells, (bar 2) LPS alone, (bar 3) LPS + celecoxib, (bar 4) LPS + isoorientin 1 μM, (bar 5) LPS + isoorientin 5 μM, (bar 6) LPS + isoorientin 10 μM, and (bar 7) LPS + isoorientin 25 μM. (b) Air pouch tissue, (bar 1) control, (bar 2) carrageenan, (bar 3) carrageenan + celecoxib, (bar 4) carrageenan + isoorientin 10 mg/Kg body weight, and (bar 5) carrageenan + isoorientin 20 mg/Kg body weight.

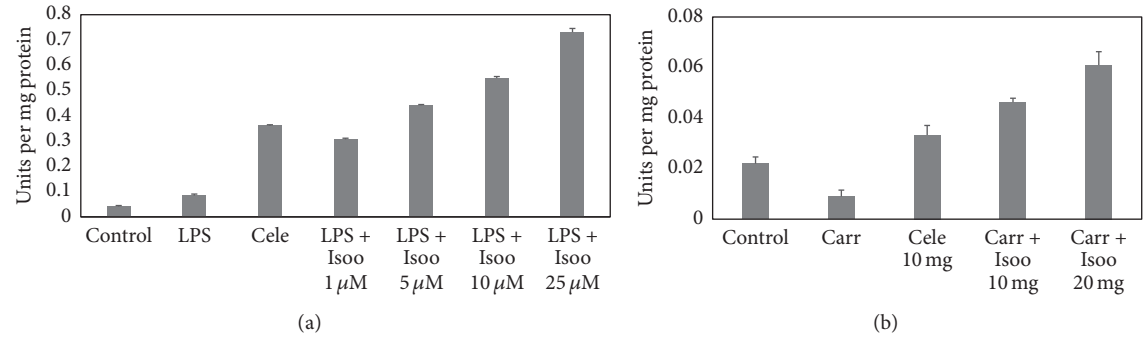

Figure 10: Effect of isoorientin on GST activity in RAW 264.7 cells and air pouch tissue. (a) RAW 264.7 cells, (bar 1) control cells, (bar 2) LPS alone, (bar 3) LPS + celecoxib, (bar 4) LPS + isoorientin 1 μM, (bar 5) LPS + isoorientin 5 μM, (bar 6) LPS + isoorientin 10 μM, and (bar 7) LPS + isoorientin 25 μM. (b) Air pouch tissue, (bar 1) control, (bar 2) carrageenan, (bar 3) carrageenan + celecoxib, (bar 4) carrageenan + isoorientin 10 mg/kg body weight, and (bar 5) carrageenan + isoorientin 20 mg/kg body weight.

dependent manner. TNF-α and IL-1β are known to have key role in inflammatory processes and are mainly produced by macrophages [19]. In the present study isoorientin treatment significantly reduces the expression of TNF-α and IL-1β in a concentration dependent manner. NF-$\kappa\beta$ is known to control the expression of cell survival genes, cytokines and proinflammatory markers [19]. Isoorientin inhibited the LPS induced translocation of NF-$\kappa\beta$/p65 as evidenced by Western blotting.

In the air pouch model the inflammatory reaction gradually progressed with time and reached a peak at 24 h after carrageenan treatment. Mice treated with isoorientin showed less inflammation compared to carrageenan treated mice. Histopathological studies clearly demonstrate that carrageenan treated pouch tissue showed heavy infiltration of blood cells at various sites in the tissue, whereas isoorientin treated animals, however, showed reduced inflammatory reaction as indicated by less degree of cellular infiltration. Carrageenan treatment increased the expression of COX-2, TNF-α, IL-1β, iNOS, and 5-LOX and this was decreased by isoorientin treatment in dose dependent manner.

Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals [20]. Reactive oxygen species (ROS) include all those reactive radical and nonradical oxygen species which are highly reactive entities and can readily participate in a variety of chemical/biochemical reactions. ROS can be formed in the heart and other tissues, by several mechanisms; they can be produced by xanthine oxidase (XO), NAD(P)H oxidase, cytochrome P450; by autooxidation of catecholamine; and by uncoupling of NO synthase (NOS) [21–24]. There are several cellular mechanisms that counterbalance the production of ROS, including enzymatic and nonenzymatic pathways [25]. Among the best-characterized enzymatic pathways are catalase and glutathione-S-transferase (GST). Nonenzymatic mechanisms include intracellular antioxidants such as the vitamins E, C, and β-carotene (a precursor to vitamin A), ubiquinone, lipoic acid, and urate [25]. We found that isoorientin is able to increase catalase activity in tissues of isoorientin treated mice. We also found that isoorientin increased GST activity that was decreased on carrageenan treatment.

6. Conclusions

Isoorientin, selective COX-2 inhibitor isolated from tubers of *P. tuberosa*, showed potent anti-inflammatory properties in vitro on mouse macrophage cell line, RAW 264.7, challenged

with LPS. Also isoorientin was found to be effective in reducing the carrageenan induced inflammation in vivo on paw edema as well as air pouch mouse models. These effects of isoorientin appear to be mediated by the inactivation of NF-κB and downregulating the expression of proinflammatory genes such as COX-2, iNOS, and TNF-α and activation of antioxidant defense enzymes such as catalase and GSTs.

Abbreviations

COX: Cyclooxygenase
COXIBS: Cyclooxygenase inhibitors
DMSO: Dimethyl sulfoxide
GST: Glutathione-S-transferase
IL: Interleukin
iNOS: Inducible nitric oxide synthase
LPS: Lipopolysaccharide
MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB: Nuclear factor kappa B
NSAIDs: Nonsteroidal anti-inflammatory drugs
TNF-α: Tumor necrosis factor-α.

Competing Interests

The authors wish to confirm that there are no known competing interests associated with this publication.

Authors' Contributions

Kotha Anilkumar and Gorla V. Reddy equally contributed.

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Research Article

The Peroxidation of Leukocytes Index Ratio Reveals the Prooxidant Effect of Green Tea Extract

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Despite tea increased plasma nonenzymatic antioxidant capacity, the European Food Safety Administration (EFSA) denied claims related to tea and its protection from oxidative damage. Furthermore, the Supplement Information Expert Committee (DSI EC) expressed some doubts on the safety of green tea extract (GTE). We performed a pilot study in order to evaluate the effect of a single dose of two capsules of a GTE supplement (200 mg × 2) on the peroxidation of leukocytes index ratio (PLIR) in relation to uric acid (UA) and ferric reducing antioxidant potential (FRAP), as well as the sample size to reach statistical significance. GTE induced a prooxidant effect on leukocytes, whereas FRAP did not change, in agreement with the EFSA and the DSI EC conclusions. Besides, our results confirm the primary role of UA in the antioxidant defences. The ratio based calculation of the PLIR reduced the sample size to reach statistical significance, compared to the resistance to an exogenous oxidative stress and to the functional capacity of oxidative burst. Therefore, PLIR could be a sensitive marker of redox status.

1. Introduction

The Supplement Information Expert Committee (DSI EC) indicated that consumption of green tea extract (GTE) could induce liver damage [1]. In fact, there are an increasing number of case reports of hepatotoxicity in humans associated with intake of green tea (GT) dietary supplements [2–7]. The types of preparation responsible for the adverse effects were hydroalcoholic extract and aqueous extract of GT, consumed as tea or in capsules [7]. However, there are also cases reporting hepatotoxicity after GT infusion [2–7]. In particular, a case has been reported with features mimicking autoimmune hepatitis, with abnormal liver histology and elevated levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, gamma glutamyl-transferase and bilirubin, associated with hypergammaglobulinemia, and the transient presence of anti-smooth-muscle antibodies (ASMA) and anti-neutrophil cytoplasmic antibodies (ANCA) [8]. GT withdrawal resulted in a slow and continuous improvement with a complete resolution after 7 months [8].

Furthermore, the Food and Drug Administration (FDA) and the European Food Safety Administration (EFSA) have denied the proposed health claims for GT and decreased risk of noncommunicable diseases [9]. In particular, despite GT increased plasma nonenzymatic antioxidant capacity (NEAC) [10], the EFSA denied claims related to tea and protection of DNA and lipids from oxidative damage [9].

GT contains several flavonoids with antioxidant properties, in particular the flavanol monomers known as catechins, where epigallocatechin-3-gallate (EGCG) is the most effective antioxidant compound [11]. However, tea catechins could have also prooxidant activity [11]. Besides, some of the protective effects of EGCG have been ascribed to its capability to reduce excessive uric acid (UA) level [12]. In particular, flavanols of *Camellia sinensis* modulate both xanthine oxidase and urate transport [13]. UA is the major plasma antioxidant and contributes to plasma nonenzymatic antioxidant capacity [10].

The peroxidation of leukocytes index ratio (PLIR) measures the resistance of leukocytes to exogenous oxidative stress and their functional capacity of oxidative burst upon activation [14].

Therefore, we performed a pilot study in order to evaluate the effect of a single dose of a GTE supplement on the PLIR,

in relation to plasma UA and ferric reducing antioxidant potential (FRAP) [15], as well as the sample size to reach statistical significance.

2. Material and Methods

2.1. Subjects and Treatment. Participants (6 men and 4 women, 19–35 years old) to the study, who volunteered in response to advertisements, were healthy, nonsmokers and were taking no supplements.

For two days prior to each feeding study the subjects followed a low antioxidant diet (washout) by avoiding all fresh fruit, vegetables, tea, coffee, cocoa, fruit juices, and wine.

On the day of the study, after an overnight fast, venous blood samples were collected (in EDTA-tubes) before (T0), 30 minutes (T0.5), and 3 hours (T3) after a single dose of two capsules of a GTE (200 mg × 2), commercially available in Italy (cod. 1820, REGISTRO INTEGRATORI https://www.salute.gov.it/imgs/C_17_pagineAree_3668_listaFile_itemName_1_file.pdf).

2.2. Plasma Uric Acid and TAC. The plasma was separated by centrifugation at 1300 ×g at 4°C for 15 min and stored at −80°C. Plasma levels of UA were quantified using colorimetric kits (Sentinel CH. SpA, Italy).

Plasma TAC was measured with the FRAP assay [15]. We calculated also the uric acid- independent FRAP (FRAP-UA) as previously described [16], applying the formula:

$$\text{FRAP-UA} = \text{FRAP } \mu\text{M} - 2\,\text{UA } \mu\text{M}. \quad (1)$$

2.3. PLIR Method. After red blood cells' lysis and 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (C11-BODIPY, Invitrogen, final concentration 1 μM) staining, leukocytes were treated as previously described [14, 17] with phorbol 12-myristate 13-acetate (PMA, Sigma, final concentration 1 μg/mL), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH, Sigma, final concentration 10 mM), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Sigma, final concentration 10 μM), PMA 1 μg/mL + Trolox 10 μM, or AAPH 10 mM + Trolox 10 μM. After 30 min at 37°C cells were stored in ice, to stop reactions, and rapidly analyzed on an Accuri C6 BD cytometer.

Data acquired on the Accuri C6 was exported in FCS format and analyzed by FCS express software (De Novo Software) to calculate the ratio of oxidation of the probe C11-BODIPY (FL1/FL2).

PLIR was calculated as previously described [14, 17], applying the formula:

$$\text{PLIR} = \frac{(\text{ratio AAPH} \times \text{ratio PMA Trolox})}{(\text{ratio AAPH Trolox} \times \text{ratio PMA})}. \quad (2)$$

2.4. Statistics. Statistical analysis, carried out with Friedman RM ANOVA on Ranks, revealed a normal distribution for all markers (Normality Test Shapiro-Wilk and Equal variance test passed).

Table 1: Effect of GTE consumption on plasma antioxidant markers and PLIR.

	T0	T0.5	T3
UA μM	334.1 ± 7.4	324.9 ± 3.9	324.3 ± 3.7
FRAP μM	1061.5 ± 38.45	1061.48 ± 52.85	1083.33 ± 46.78
FRAP-UA μM	393.23 ± 41.63*	411,57 ± 53.38	434.59 ± 41,75*
PLIR L	2.09 ± 0.17	2.09 ± 0.17	2.37 ± 0.19
PLIR M	1.88 ± 0.13*	1.86 ± 0.12	2.19 ± 0.15*
PLIR G	1.80 ± 0.12*	1.90 ± 0.14	2.17 ± 0.15*

Plasma antioxidant markers in samples collected from 10 healthy subjects before (T0), 0.5 (T0.5), and 3 hours (T3) after the consumption of a single dose of two capsules of a green tea extract (GTE) supplement (200 mg × 2). UA: uric acid, FRAP: ferric reducing antioxidant potential, FRAP-UA: uric acid-independent FRAP, PLIR: peroxidation of leukocytes index ratio, L: lymphocytes, M: monocytes, and G: granulocytes. RM ANOVA, with time as within-subjects factor, followed by Student-Newman-Keuls post hoc analysis: T3 versus T0: $^*p < 0.05$.

Therefore, statistical analysis was carried out with repeated measures analysis of variance (RM ANOVA), with time or treatment as within-subjects factors. Student-Newman-Keuls post hoc analysis (all pairwise multiple comparison procedure) was used to isolate differences between groups. Spearman correlation was used to evaluate relationships between variables. All statistical evaluations were performed using the SigmaStat and SigmaPlot software (Jandel Scientific, Inc.).

3. Results

3.1. Plasma Uric Acid and TAC. RM ANOVA, with time as within-subjects factor, followed by Student-Newman-Keuls post hoc analysis (all pairwise multiple comparison procedure), revealed that GTE consumption did not affect FRAP values, whereas 3 hours (T3) after treatment both a nonsignificant decrease in UA and a significant increase in FRAP-UA were found (Table 1).

From the difference of means and the standard deviations (power 0.8; alpha 0.05) we calculated a sample size (to reach statistical significance) of 106 for UA.

3.2. PLIR Method. Treatment with GTE significantly increased PLIR of monocytes and granulocytes at T3, whereas a nonsignificant increase was observed for PLIR of lymphocytes (Table 1). We calculated a sample size (to reach statistical significance) of 80 for PLIR of lymphocytes.

Pearson Product Moment Correlation revealed an inverse correlation of UA with PLIR L (CC = −0.383, $p = 0.0368$), PLIR M (CC = −0.474, $p = 0.008$), and PLIR G (CC = −0.545, $p = 0.001$) and a direct correlation of FRAP-UA with PLIR L (CC = 0.451, $p = 0.012$), PLIR M (CC = 0.398, $p = 0.029$), and PLIR G (CC = 0.434, $p = 0.016$).

3.3. Ratio of Oxidation of the Probe C11-BODIPY. Typical overlay dot plots of the four treatments used for PLIR calculation and ratio of fluorescence (FL1/FL2) on single cells, before GTE consumption and 3 hours after, are presented in Figures 1 and 2, respectively.

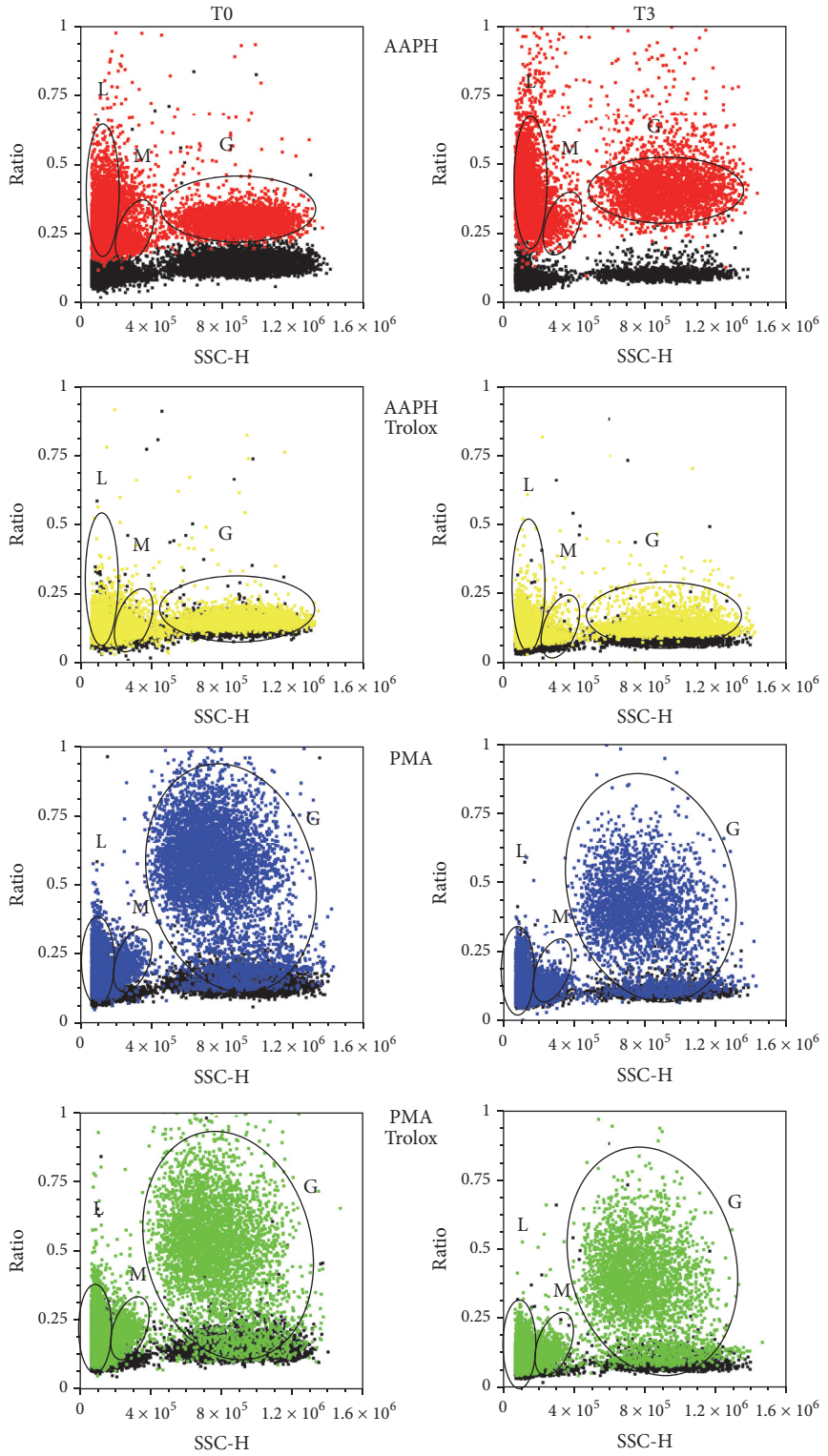

Figure 1: Typical overlay dot plots of ratio (ratio of oxidation of the probe C11-BODIPY: FL1/FL2) versus side scatter (SSC): before (T0) and 3 hours (T3) in leukocytes collected after a single dose of two capsules of a green tea extract (GTE) supplement (200 mg × 2). L: lymphocytes, M: monocytes, and G: granulocytes. Unstimulated samples (black) and leukocytes treated with 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH, 10 mM, red), AAPH (10 mM) + 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 10 μM, yellow), phorbol 12-myristate 13-acetate (PMA, 1 μg/mL, blue), or PMA (1 μg/mL) + Trolox (green).

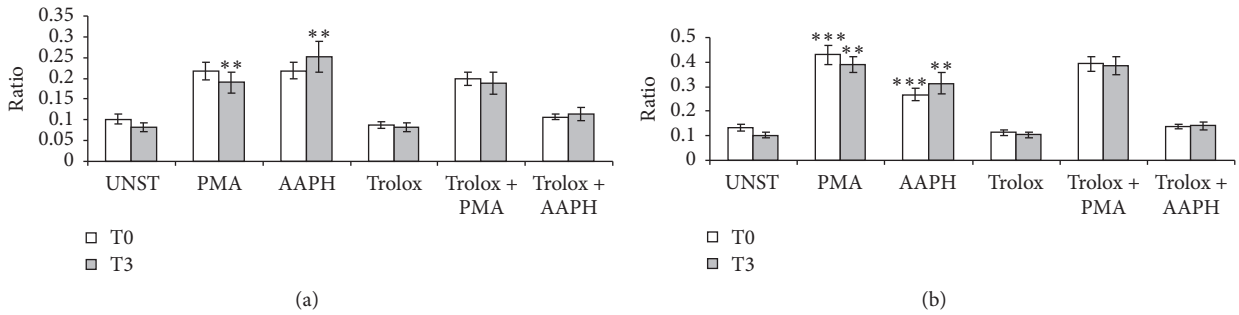

Figure 2: Ratio (ratio of oxidation of the probe C11-BODIPY: FL1/FL2) of monocytes (a) and granulocytes (b), in samples collected from 10 healthy subjects before (T0) and 3 hours (T3) after the consumption of a single dose of two capsules of a green tea extract (GTE) supplement (200 mg × 2). Cells unstimulated (UNST) or treated with phorbol 12-myristate 13-acetate (PMA, 1 μg/mL), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH, 10 mM), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 10 μM), Trolox + PMA, or Trolox + AAPH. Two-way (2W) RM ANOVA, with time and treatment as within-subjects factors, followed by Student-Newman-Keuls post hoc analysis: AAPH versus PMA within time: $^{**}p < 0.01$, $^{***}p < 0.001$.

Trolox inhibited the peroxidation of C11-BODIPY in leukocytes exposed to AAPH free radicals generating system, but not the PMA-induced oxidation in monocytes and granulocytes, both at T3 and at T0 (Figure 1).

Considering the major components of PLIR affected by treatment, compared to baseline, the AAPH-induced (exogenous) oxidation appeared greater, whereas the PMA-induced oxidative burst appeared lower (Figure 1).

Despite the differences of ratio PMA and ratio AAPH between times did not reach significance, the statistical significance between ratio AAPH and ratio PMA was different at baseline and at T3 on granulocytes and monocytes (Figure 2). We calculated a sample size (to reach statistical significance between times) of 17 and 33 for ratio AAPH and of 21 and 51 for ratio PMA, for granulocytes and monocytes, respectively.

Ratio PMA was not related to neither UA nor FRAP-UA, whereas ratio AAPH was inversely correlated with UA on all cells (L: CC = −0.477, $p = 0.007$; M: CC = −0.514, $p = 0.003$; G: CC = −0.511, $p = 0.003$), but not with FRAP-UA.

4. Discussion

4.1. Effect of GTE on Plasma Antioxidants. Previous studies reported decreased, increased, or unchanged UA and NEAC levels after bolus consumption of EGCG or GTE [18–22]. EGCG increased in plasma from 30 min to 2.6 hours after GTE consumption, depending on the dose and on the formulation [21–25]. However, the FRAP value did not increase when free EGCG concentration was at its peak [21], probably due to the decrease in UA levels observed after GTE consumption [22].

In agreement with these results, in our study GTE consumption did not affect FRAP values whereas a nonsignificant decrease in UA and a significant increase in FRAP-UA were found 3 hours after treatment. The increase in FRAP-UA, probably due to the catechins, could counterbalance the reduction in FRAP induced by the UA decrease. However, the FRAP assay matches the antioxidant capacity to the reducing ability [15] and the reduced iron is critical in the onset of oxidative stress due to the Fenton reaction that generates the hydroxyl radical initiator of lipid peroxidation [26].

Therefore, an increase in the metal reducing power could be more likely detrimental than beneficial.

4.2. Effect of GTE on AAPH-Induced Lipoperoxidation. The increase in FRAP-UA was temporally associated with an increased oxidation of the fluorescent probe C11-BODIPY incorporated into the leukocytes. In this context, the prooxidant effects of tea catechins on cells are supported by the molecular mechanisms involved in their induction of antioxidant enzymes, through the antioxidant responsive elements (ARE) pathway [11, 27]. In particular, it has been suggested that some derivatives of catechins can oxidize highly reactive cysteine thiol groups of Kelch-like ECH-associated protein-1 (Keap1), resulting in disulfide bond formation and nuclear factor-erythroid 2-related factor 2 (Nrf2) release [27].

However, the ratio AAPH was inversely correlated with UA on all cells, but not with FRAP-UA. In agreement with our results the consumption for 112 days of a lutein (12 mg/d) plus GTE (200 mg/d) supplement did not reduce the oxidation of the C11-BODIY incorporated into the lipid compartment of plasma [28].

4.3. Effect of GTE on PMA-Induced Oxidative Burst. We have found a nonsignificant decrease of PMA-induced lipoperoxidation after GTE consumption, contrarily with the increase of the oxidative burst of granulocyte, observed in cyclists when quercetin was administered with the tea flavanol epigallocatechin 3-gallate, by using dihydrorhodamine 123 (DHR123) as fluorescence probe [29]. However, the post-exercise-induced decrease in oxidative burst was unaffected after bolus consumption of the same supplement [29, 30], when hydroethidine was used as probe [30]. Therefore our results confirm that the plasma membrane C-11 BODIPY is a suitable probe in the evaluation of the effects on the oxidative burst of flavonoids, which increase DHR123 accumulation [31]. Though the effect of GTE consumption on oxidative burst requires more subjects to reach statistical significance, our results are in agreement with the reduction of the p22phox subunit of the NADPH oxidase observed in hemodialysis patients after 6 months of treatment with GT [32].

4.4. Effect of GTE on PLIR. In a postprandial study [17], we observed that a functional food covered by dark chocolate and containing glucomannan, inulin, fructooligosaccharides, and Bacillus coagulans strain GanedenBC30 significantly improved postprandial metabolic stress (insulin, glucose, and triglycerides), reduced the postprandial increase of UA, and improved PLIR of lymphocytes, but not of monocytes and granulocytes. We suggested that, although PLIR is a functional index that is independent of baseline levels of oxidation, measuring the ratio between the resistance to exogenous and the resistance to endogenous ROS injury, this ratio calculation could mask the effect of foods that inhibit both the exogenous ROS injury and the oxidative burst [17].

On the contrary, in the present study, treatment with GTE significantly increased PLIR of monocytes and granulocytes at T3 after ingestion, whereas a nonsignificant increase was observed for PLIR of lymphocytes. An inverse correlation of UA with PLIR and a direct correlation of FRAP-UA with PLIR of all leukocytes were found. Therefore, though some of the protective effects of catechins have been ascribed to their capability to reduce excessive UA level [12], in our study the inverse correlation of PLIR with UA levels, in particular with the ratio AAPH component of PLIR, confirms that UA is a major circulating antioxidant as suggested by Fabbrini et al. [33]. Authors [33] reported that rasburicase treatment, in subjects who had high serum UA concentrations, caused a marked decrease in plasma FRAP and a significant increase in urinary isoprostanes/creatinine ratio and in skeletal muscle protein carbonylation.

On the other hand, though the effects of GTE consumption on oxidative burst and AAPH-induced lipoperoxidation require more subjects to reach statistical significance, the statistical significance between ratio AAPH and ratio PMA was different at baseline and at T3 on granulocytes and monocytes. Therefore, the ratio based calculation of the PLIR is able to appreciate differences also with a low number of subjects in monocytes and granulocytes.

5. Conclusion

In conclusion our study suggests that PLIR, in particular PLIR of granulocytes where the differences between ratio AAPH and ratio PMA are more evident, reveals the prooxidant effect of GTE. The direct correlation of FRAP-UA with PLIR suggests that the iron reducing power of GTE could be more likely detrimental than beneficial. This result is in agreement with the FDA, the EFSA, and the DSI EC conclusions [1, 9]. Furthermore, the inverse correlation of UA with PLIR confirms the primary role of UA in the antioxidant defences. Therefore, PLIR could be a sensitive marker of redox status.

Abbreviations

AAPH: 2,2′-Azobis(2-methylpropionamidine) dihydrochloride
C11-BODIPY: 4,4-Difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid
DHR123: Dihydrorhodamine 123
DSI EC: Supplement Information Expert Committee
EFSA: European Food Safety Administration
FDA: Food and Drug Administration
FRAP: Ferric reducing antioxidant potential
GT: Green tea
GTE: Green tea extract
NEAC: Nonenzymatic antioxidant capacity
PLIR: Peroxidation of leukocytes index ratio
PMA: Phorbol 12-myristate 13-acetate
Trolox: 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
UA: Uric acid.

Ethical Approval

Approval for the study was obtained from the Ethics Committee for Human Non-Clinical Research of the Department of Physiology and Pharmacology "V. Erspamer," "Sapienza," University of Rome, and all procedures involving human subjects complied with the Declaration of Helsinki as revised in 2000.

Consent

Written informed consent was obtained from all the participants in accordance with the Italian law (law number 196/2003, Ministry of Health Circular Letter GU number 76/2008).

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contributions

Ilaria Peluso designed the research, analyzed the data, and drafted the paper. Husseen Manafikhi, Anna Raguzzini, and Raffaella Reggi performed the analyses. Yaroslava Longhitano and Christian Zanza performed the blood sampling. Maura Palmery critically reviewed the paper and supervised the whole project.

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