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CORSO DI LAUREA (MAGISTRALE) IN SCIENZE DELL'ALIMENTAZIONE E DELLA NUTRIZIONE UMANA

Effects of Opuntia Ficus Indica waste material on GSNOR expression: mechanisms and possible applications

Relatrice
Prof.ssa Barbara Benassi

Correlatore
Prof.re Giuseppe Filomeni

Laureanda
Valeria Zanghi Buffi

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INTRODUCTION

1. CANCER

Cancer is considered one of the major leading causes of death worldwide. The etiology of cancer depends on both environmental factors (accounting for the 90% of cases) and genetic inheritance. Among the environmental factors, lifestyle (smoking, diet, UV radiation) is undoubtedly the one that plays the major role, with viral infections and chemical exposure being marginal. Nowadays, breast, lung, prostate and colorectal cancer account for the highest mortality rates in the world. (3) According to the American Cancer Society statistics, cases are expected to rise with 23.6 million new cancer cases by 2030. It is, therefore, important to evaluate the mechanisms involved in tumor initiation and progression to develop preventive and therapeutic measures.

1.1 COLORECTAL CANCER: PATHOGENESIS AND EPIDEMIOLOGY

According to GLOBOCAN 2020 data, colorectal cancer (CRC) is the third most incident cancer in the world. The estimated number of cases worldwide for both sexes and for all ages (excl. NMSC) is about 1.931.590 (Figure 1).

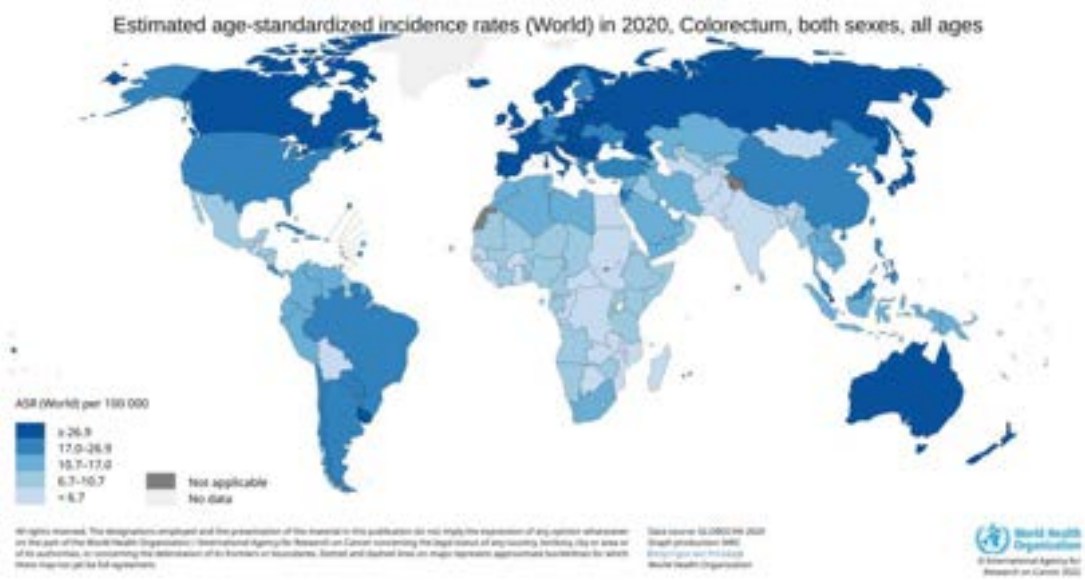

Figure 1. Map showing estimated age-standardised incidence rates (world) in 2020, colorectum, both sexes, all ages

CRC is more incident among men than women and three to four-times more common in Western Countries than in developing nations. Age-standardised (world) incidence rates per 100.000 of CRC is 19.7 in both sexes (Figure 2), 23.4 in males 16.2 in females[1]. (1)

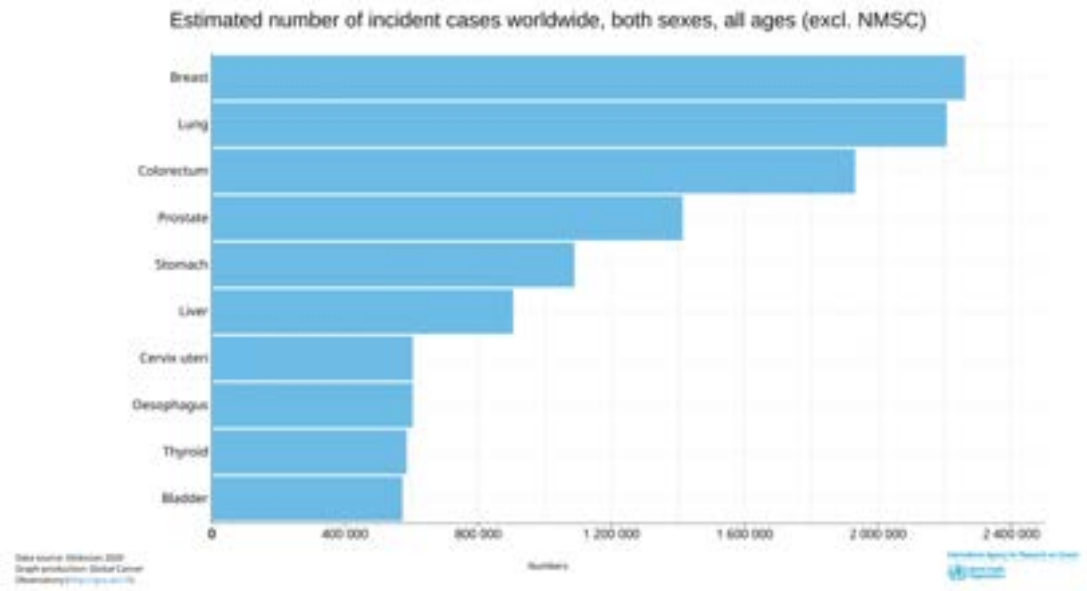

Figure 2. Bar chart showing Age-standardised (world) incidence rates per 100,000 of CRC in both sexes (http://globocan.iarc.fr)

In the last few years, improvements in CRC treatment have led to decreases in colorectal cancer mortality. Driver has been the removal of polyps and other early detection efforts, such as colonoscopies, flexible sigmoidoscopies, computed tomography (CT) colonography, faecal immunochemistry, and faecal occult blood testing. The introduction of better screening tests may have initially increased incidence rates due to the diagnosis of previously undiagnosed disease and, in the long term, has reduced mortality thanks to the removal of pre-cancerous or un-metastasised polyps (2).

[1] All data are from: https://gco.iarc.fr

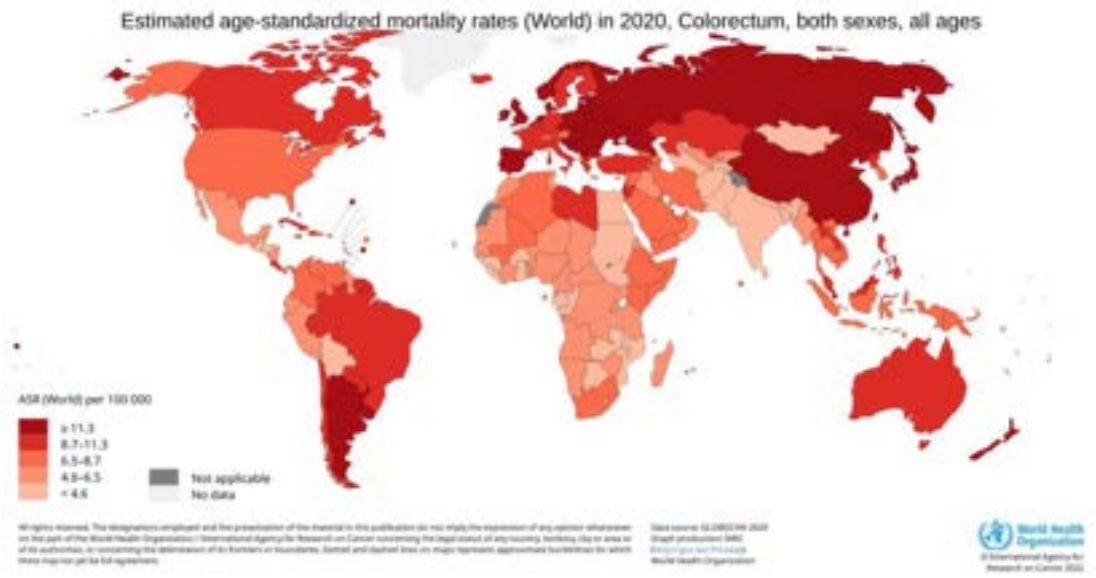

Figure 3. Map showing estimated age-standardised mortality rates (world) in 2020, colorectum, both sexes, all ages (reproduced from http://globocan.iarc.fr)

Notwithstanding the progress done in cancer screening and treatments, a still great number of patients develops cancer due to unclear reasons. Many lines of research of the last few decades have suggested that nutrition may play both a causal and protective role in the development of colon cancer (3). Indeed, epidemiological and experimental data have linked the consumption of several foods and nutrients to the risk of colorectal neoplasia (4). Different lines of evidence suggest that a combination of phytochemicals, nutraceuticals, diet and exercise could prevent the risk of disease (5). Indeed, the use of polyphenols and phytochemicals – a class of biomolecules known as potent micronutrients – has been proposed as promising approach in the prevention of various diseases, such as diabetes, cardiovascular diseases, and cancer (2). This biomolecules can indeed modulate, directly or indirectly, cell signaling and protein posttranslational modification (PTMs), such as phosphorylation and acetylation (6). Very recently it has been proposed that the reversible PTM induced by nitric oxide (NO), so called S-nitrosylation, is linked to cancer and can be affected by phytochemicals (3).

2. NITRIC OXIDE

Nitric oxide (NO) is a short-lived, highly reactive gaseous molecule ubiquitously produced in vertebrates. Nitric oxide acts as a signal molecule capable of regulating gene transcription, neuronal activity, blood pressure, and other physiological processes (7). In cells, NO is produced upon the enzymatic oxidation of L-arginine into citrulline by nitric oxide synthases (NOSs). There are three different NOSs, two of which are constitutively expressed – predominantly in neurones (nNOS) and endothelial tissue (eNOS) – and a third one, inducible (iNOS), predominantly expressed in immune cells and in other tissue during the immune response (8) (Table 1). Nitric oxide has different biological activities and can react with a number of molecules, e.g. superoxide, forming peroxynitrite, which can mediate bactericidal or cytotoxic reactions. NO also mediates smooth muscle relaxation, neurotransmission, and modulation of inflammation in a number of organ systems and pathophysiologic conditions (9).

Table 1. Genes for human NOS isoforms

Human NOS isoform	**Genbank accession numbers**
nNOS (NOS1)	L02881,U11422
iNOS (NOS2)	LO9210,L24553,X73029
eNOS (NOS3)	M93718,M95296

Nitric oxide is hydrophobic. Therefore, it can pass easily through membranes but, due to its high reactivity it persists in vivo for a few seconds, and can diffuse several cell diameters from its site of synthesis. Nitric oxide and its products react with a large number of biological molecules: NO binds directly to heme-iron, forming iron-nitrosyl complexes in vivo. This leads to inhibition of several haem-containing enzymes such as cytochrome c oxidase, catalase, and cytochrome P-450. Iron-sulphur (FeS) proteins are also particularly sensitive to NO, mostly to the detrimental effect of peroxynitrite a highly reactive and damaging NO derivative (8).

3. NITROSATIVE AND OXIDATIVE STRESS

Oxidative stress is a condition established by an imbalance of pro-oxidant versus antioxidant molecules, in favour of the first. Oxidative stress can produce damage biomolecules and cell structures (6). Pro-oxidant species are a group of inorganic and highly reactive molecules that can oxidize and alter carbohydrates, nucleic acids, lipids, and proteins (10). These molecules derive from different chemical elements as carbon, nitrogen, sulphur. However, oxidative stress is generally described as an excessive or deregulated production of radical and non-radical species derived from oxygen, called reactive oxygen species (ROS), which oxidize proteins, lipids, carbohydrates and nucleotides (11). The high chemical reactivity of ROS makes them very effective "weapons" against most biomolecules. They are implicated in an increasing number of varied and dynamic processes in molecular and cell biology research, whose underlying chemical pathways are often unknown (12).

Similarly to oxygen, also nitric oxide can form reactive species, usually referred to as reactive nitrogen species (RNS). RNS are a group of molecules with different properties and reactivity. Some RNS are highly reactive and typically induce irreversible modifications, which suggest they lack the specificity and "reversibility" required for cell signaling events (13). Conditions in which NO is overproduced are referred to nitrosative stress.

Nitrosative stress is the major cause of damage, which is largely associated to the reaction of NO with superoxide ($O_2^{\cdot -}$) to form peroxynitrite ($ONOO^-$). $ONOO^-$ is, indeed, highly instable with a half-life < 1 s this feature making it more reactive and damaging than its precursors (8).

Peroxynitrite and other RNS are formed upon an abnormal increase in the level of NO and $O_2^{\cdot -}$. The irreversible damage that they induce to cellular biomolecules have been involved in cell death (14) and in the pathogenesis of many diseases correlated with inflammatory processes and/or neurotoxicity (15).

4. S-NITROSYLATION

In cells, NO mediates its manifold biological via different post-translational modification, among which *S*-nitrosylation plays a major role. *S*-nitrosylation is a reversible post-translational modification of proteins characterized by the covalent binding of NO to cysteine residues of proteins and low-molecular weight molecules (such as glutathione) (16–18). Similarly to other post-translational modifications, *S*-nitrosylation has also been found to regulate a broad range of biologic, physiologic and cellular functions (19).

From a chemical point of view, *S*-nitrosylation is the covalent addition of an NO group to a reactive sulfhydryl, resulting in the formation of *S*-nitrosothiols (SNOs) (Figure 4). SNOs are divided in: i) low-molecular weight SNOs (LMW-SNOs), such as S-nitrosocysteine (SNOC) or S-nitrosoglutathione (GSNO), and ii) S-nitrosylated proteins (PSNOs). The generation of PSNOs strongly depends on the protein conformation and the chemical microenvironment of the cysteine residues (20). It has been proposed that the specificity of the SNO site is based, in fact, on the presence of a consensus motif of *S*-nitrosylation. This motif does not consist of a true consensus sequence, but rather in specific physical-chemical properties of the protein environment surrounding the S-nitrosylation site (21, 22).

Protein *S*-nitrosylation occurs physiologically and it is nowadays widely accepted that it represents the key signaling mechanism underlying NO bioactivity. Numerous cellular processes including cell cycle progression, metabolism, transcription, DNA repair and programmed cell death have been reported to be regulated by NO through *S*-nitrosylation (23, 24). Consequently, a disbalance between NO production and protein denitrosylation (the chemical reaction that terminates the signal) is associated with several pathological states, including cancer (23). *S*-nitrosylation plays a central role in propagating NO signals within a cell, tissue, and tissue microenvironment, as the nitrosyl moiety can rapidly be transferred from one protein to another. This modification has also been reported to confer either tumor-suppressing or tumor-promoting effects and is portrayed as a process involved in every stage of cancer progression (25). Nitric oxide fluxes, indeed, can activate pro-tumorigenic pathways and inhibit cell death. This dual mechanism of action depends on different causes such

as: i) NO production rate; ii) NO-producing cell type; iii) cellular compartments in which NO biosynthesis takes place; iv) timing of NO signal over tumor progression (23). Excessive *S*-nitrosylation can be result of increased expression/activity of NOSs and/or decreases/mutations in enzymes aimed at catabolizing *S*-nitrosylated cysteines, so called denitrosylases. The up-regulation of iNOS is the most common cancer-related alteration responsible for the activation of oncogenic pathways by *S*-nitrosylation (25). However, other NOS isoforms have been found to be dysregulated in cancer (26). Similarly, the down-regulation of the main denitrosylating enzymes, S-nitrosoglutathione reductase (GSNOR) and thioredoxin (Trx) — which causes the accumulation of S-nitrosylated proteins (PSNOs) — have been linked to tumor induction and progression (23). Many recent pieces of evidence have indicated that dysregulation of S-nitrosylation can lead to severe pathological events, including cancer onset, progression, and treatment resistance. The S-nitrosylation status may be directly linked to many cancer therapy outcomes as well as therapeutic-resistance.(27)

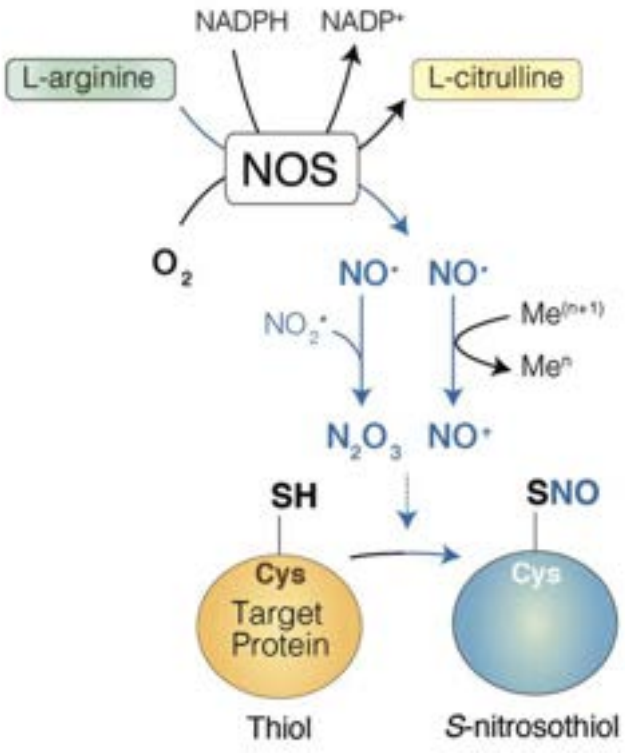

Figure 4: Nitric oxide (NO·) is a radical species produced by nitric oxide synthase (NOS), a family of NADPH-dependent enzymes, through a reaction that uses L-arginine and molecular oxygen (O2) as substrates (top). Upon reaction with nitrogen dioxide (NO2.) or redox metals (e.g., Fe3+, Cu2+), NO

gives rise to dinitrogen trioxide (N2O3) or nitrosonium ion (NO+), respectively. These species can easily react with cysteine residues of proteins (SH) to generate an S-nitrosothiol (SNO), thus changing the protein's chemical and physical properties (e.g., function, activity, stability). A distinctive feature of protein S-nitrosylation is that the NO moiety can be transferred by trans-nitrosylation to other protein or nonprotein thiols, rapidly conveying the signal without the use of energy-consuming processes. (28)

5. DE-NITROSYLATION

As above mentioned, *S*-nitrosylation is a reversible reaction. Mammalian cells have, indeed, developed (and evolutionarily conserved) enzymatic systems able to catalyze the opposite reaction, so called denitrosylation, and, in doing so, terminate NO signaling.[2]

The main mechanism responsible for protein denitrosylation is based on the chemical transfer of the NO group from S-nitrosylated proteins (PSNO) to low-molecular weight thiols, such as glutathione (GSH) and Coenzyme A (CoA) in a reaction called *trans*-nitrosylation, which generates S-nitrosoglutathione (GSNO) and S-nitroso-CoA (SNO-CoA). Intracellularly, GSNO and SNO-CoA levels are controlled by two enymes: S-nitrosoglutathione reductase (GSNOR) and SNO-CoA reductase (SCoR) (Figure 5). Therefore, by denitrosylating their substrates, GSNOR and SCoR, indirectly regulate PSNO levels. (29)

2 Like the phosphatases protein that remove the phosphoric group (PO32-); deubiquitinases, which remove ubiquitin monomers, or demethylases which detach, through oxidative decarboxylation processes, the methyl group (CH3) from DNA or histones.

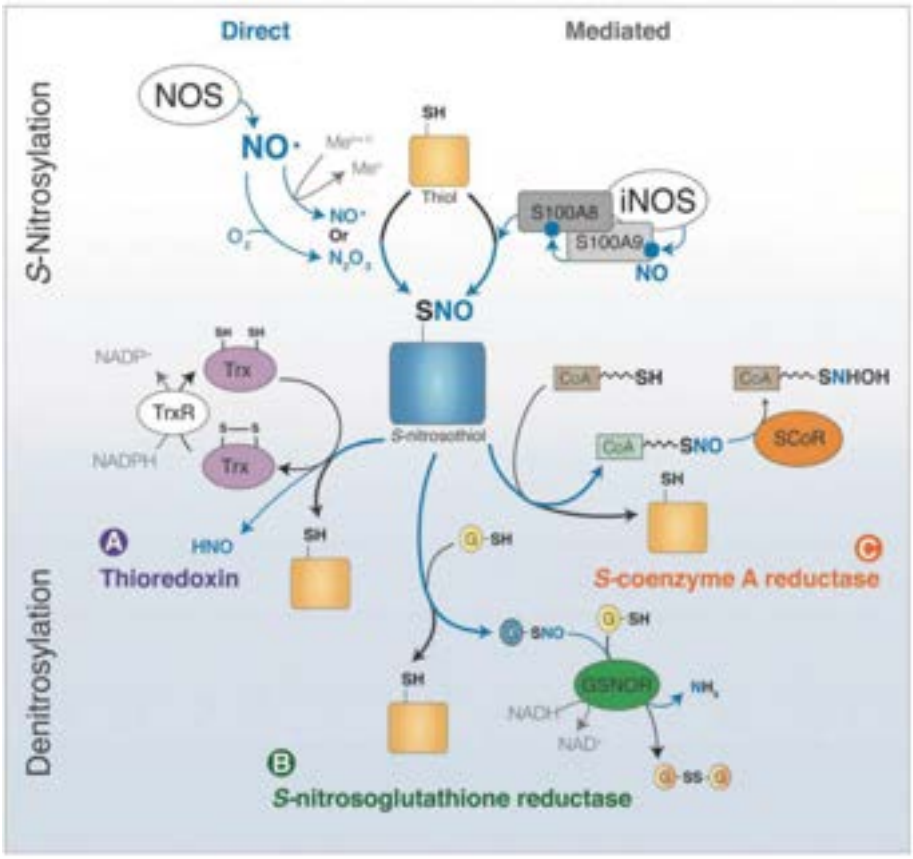

Figure 5: Protein S-nitrosylation and denitrosylation (20)

6. GSH-GSNOR SYSTEM

A distinctive feature of S-nitrosylation is that NO moiety can be transferred by *trans*-nitrosylation to other protein or non-protein thiols. Therefore, S-nitrosylated proteins are in equilibrium with GSH, the most abundant cellular thiol, thus leading to its conversion into S-nitrosoglutathione. In this way, PSNOs levels are modulated by GSNO and vice versa. In 1998, Jensen and colleagues discovered an NAD(P)H-dependent oxidoreductase that was able to catalyse the reduction of GSNO (and so named GSNO reductase, or GSNOR), leading to the formation of glutathione disulfide (GSSG) and ammonium (31). GSNOR is evolutionarily conserved from bacteria to humans, and it is ubiquitously expressed in different tissues, with the highest in brain, liver, kidney and testis (30). After purification, Jensen and colleagues discovered that this enzyme was the class III alcohol dehydrogenase (ADH III), also referred to as

GSH-dependent formaldehyde dehydrogenase (GSH-FDH). (32) As a member of ADH enzymes, GSNOR catalyzes the $NAD(P)^+$-dependent oxidation of S-hydroxymethylglutathione ($GSCH_2OH$), the intermediate generated by the spontaneous and reversible interaction between GSH and formaldehyde. Biochemical characterization of GSNOR activities highlighted different degree of specificity depending on the role: dehydrogenase or reductase. However, GSNOR activity and specificity for GSNO is higher than for $GSCH_2OH$. Moreover, reductase activity is virtually undetectable towards other GSNO-related SNOs. Likewise, the degradation rate of SNOcysteinyl-glycine and γ-glutamyl-SNOcysteine (namely, the nitroso derivatives of GSH metabolites) represents only the 1% of the rate observed for GSNO metabolism, indicating an extreme specificity of GSNOR for its natural substrate GSNO (31).

In 2001, Liu and colleagues demonstrated that deleting the GSNOR gene (*adh5*) in yeast and mice abolished the GSNO consumption and increased the intracellular concentration of both GSNO and PSNOs. In addition, mutant yeasts exhibit increased nitrosative sensitivity while maintaining their ability to withstand oxidative stress. The scientists came to the conclusion that GSNOR is essential for SNO homeostasis, protects against nitrosative stress, and is evolutionarily conserved from bacteria to humans. (33)

Thus, GSNOR, together with GSH, constitutes a system that control the protein *S*-nitrosylation (Figure 6) (25). GSNOR expression and/or activity is, indeed, directly correlated with dysfunctional *S*-nitrosylation signaling and, eventually, with pathological states such as neurodegeneration (32,33), diabetes (34,35) and cancer (23,27,36). It has been discovered that mice deficient for GSNOR ($Adh5^{-/-}$) spontaneously develop liver cancer. In the last decade, different lines of evidence have suggested a link between GSNOR and tumor progression, arguing for GSNOR as a tumor suppressor. The identification of GSNOR mechanisms that drive tumor progression, could be important in cancer research to propose new anticancer therapies aimed at restoring/reactivating GSNOR activity and targeting *S*-nitrosylation.

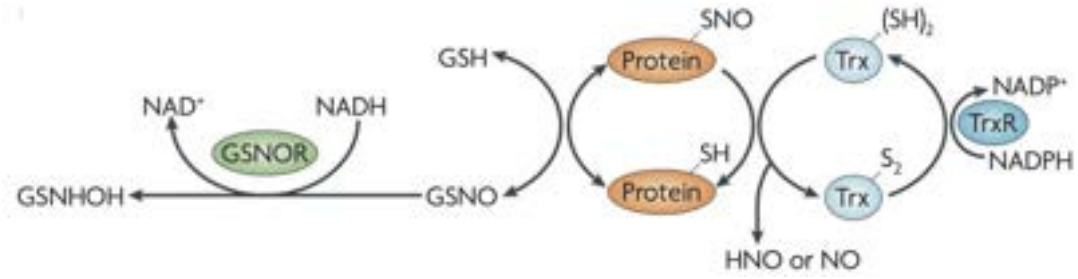

Figure 6. An SNO protein can be denitrosylated by glutathione (GSH), forming a reduced protein thiol and GSNO, of which GSNO is rapidly and irreversibly metabolized by GSNOR to GSNHOH (an N-hydroxysulphenamide). GSNOR thus lowers SNO protein levels by driving the equilibrium from SNO proteins towards GSNO (37)**.**

7. OPUNTIA FICUS INDICA

Opuntia ficus-indica (OFI) is a species native to Mexico, commonly known as *prickly pear*, belonging to the family Cactaceae. (38) This plant is mainly formed by three elements: the flower, the fruit, and the cladodes (pads) indicated by the arrows in Figure 7. The stem, segmented, can reach a height of 5-7 meters. The branches are modified into cladodes (leathery with the appearance and function of the leaf) elliptical, to bovate or oblanceolate shape in different species. The thorns can be sparse, isolated, ranging in sizes. The flowers are often terminal, yellow/orange to red and purple, shaped like a cup. The fruit is a berry, thorny, green then yellow, orange, red or purple dependent on variety, edible and therefore commercialized. (39)

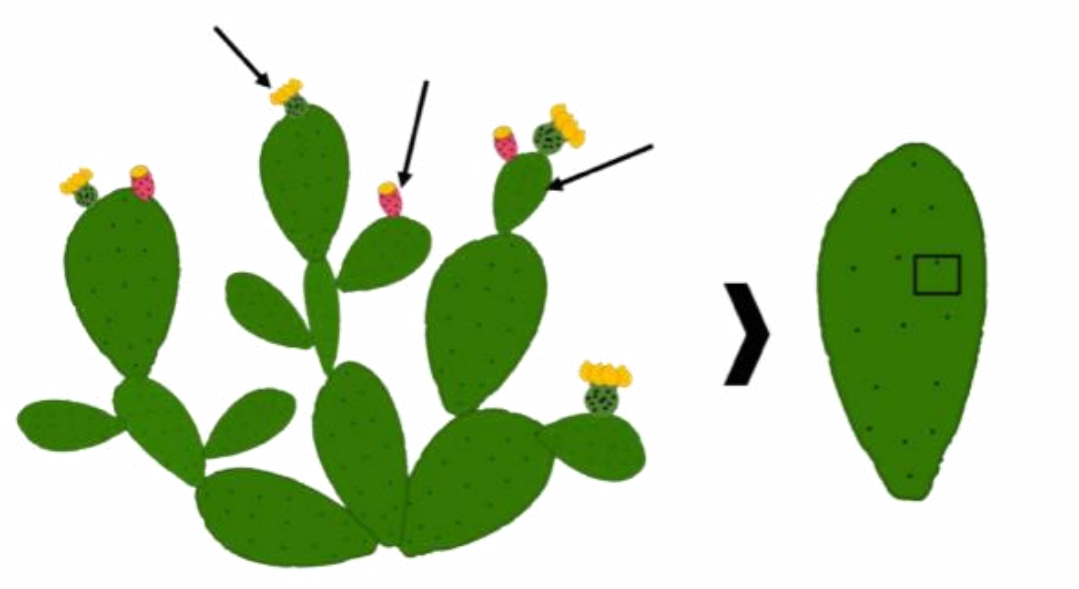

Figure 7: *Opuntia Ficus-indica* Pruning Waste Recycling: Recovery and Characterisation of Mucilage from Cladodes (2021)

OFI mucilage is a natural polymer produced in mucilage cells of parenchyma, inside the tissues of different organs (fruits and cladodes) with the main role to retain water. This mucilage is a hetero-polysaccharide of high molecular weight (2.3×10^4 - 3×10^6 $g \cdot mol^{-1}$) which consists mainly of six sugars (arabinose, galactose, rhamnose,

xylose, uronic acid and galacturonic acid), proteins and biomolecules. (40) Based on the available literature, chemical composition and properties of *Opuntia ficus-indica* mucilage from cladodes could vary, depending on season collection, age of the plant, growing conditions and different methods of extraction. (35) The economic importance of mucilage lies in its gelling properties with a multifunctional use in food, cosmetics and pharmaceutical industry. (34)

Recently, Rocchetti et al. (41) provided an analytical study on the nutraceutical composition of cladodes from "Ficodindia di San Cono", protected by designation of origin (PDO). Their results showed this "by-product" as a rich source of bioactive compounds with health-promoting properties. (35)

The cactus cladodes contain high amounts of fiber, including pectin, mucilage, lignin, cellulose and hemicellulose, and generally these substances are able to bring wellbeing to the metabolism of lipids and sugars. In particular, β-polysaccharides (i.e., glucose units linked (1→4)-β (as in cellulose) but interspersed with (1→3)-β-linkages), are characterized by an irregular linkage structure that prevents the formation of a crystalline structure leading to a water-soluble capacity. These polysaccharides are generally classified as soluble dietary fiber, improving glucose control and modulate renal water and sodium handling in type 2 diabetes patients; therefore, the high dietary fiber content of cladodes has the capacity to absorb large amounts of water, forming viscous or gelatinous colloids, and determining the absorption of several kinds of organic molecules. (41) *Opuntia ficus-indica* cladodes can also be considered a rich source of bioactive and functional compounds, which make them an important candidate for the production of health-promoting and functional foods. In this regard, in recent years, the scientific world has paid particular attention to polyphenols as they have shown antioxidant properties in vitro, together with protective effects against cancer, and the ability to cure and prevent cardiovascular disorders, inflammatory and allergic diseases. (42) Given that, is it possible to define *Opuntia* spp. as novel by-products with antioxidant activity and bioactive properties.

However, although traditionally used as a valuable health supporting nutrient, the vegetative parts of *Opuntia* spp. plants (the non-edible parts) have been scarcely

studied, and nowadays there is a lack of information on their entire chemical and bioactive properties. Nevertheless, the last scientific studies demonstrated cladodes nutritional and functional value, hence there is a growing demand for *O. ficus indica* consumption as vegetable in US and Canada, where the product reaches sales equivalent to 31 and 2.86 million dollars, respectively . (38) Furthermore, many studies based on clinical trials documented its antihyperlipidemic, antiobesity and hypocholesterolemic activity and properties. (43)

7.1 Opuntia Ficus Indica's gel: an innovative matrix

The interest in the extraction and use of this bio-product is based on the fact that the cladodes represent a pruning waste, which for example in Sicily, during the 'scozzolatura' (specialized pruning for the production of later fruits), leads to the production of about 6-10 tons / hectogram of material that must be disposed of by the farmer. The content of this innovative matrix lends itself to different uses in different fields. (44)

The chemical composition of prickly pear and cladodes depends on many factors: species, cultivar, or variety; environmental factors, such as the climatic and edaphic conditions, crop management, including fertilization and postharvest treatment and maturity status. Generally, cactus plants are important sources of bioactive substances and excellent candidates for nutraceutical and functional food preparation. Several authors confirm that prickly pear has a high bioactive potential, being an important source of bioactive com- pounds and an excellent source of dietary antioxidants, which may have beneficial effects on consumers's health (45)

In 2018 an analytical study was performed to investigate, for the first time, the presence of antioxidant constituents and the corresponding in vitro antioxidant activity in the extract of cladodes from Ficodindia di San Cono (*Opuntia ficus-indica*) protected designation of origin (PDO). According to their analysis interestingly, over 2 g/kg of polyphenols were detected in this matrix, and these compounds were mainly responsible for the antioxidant properties, as shown by the strong correlation between phenolic classes and antioxidant scores. (41)

An untargeted UHPLC-ESI/QTOF-MS approach was used to investigate the entire phenolic profile in cladodes extracts. (Figure 8)

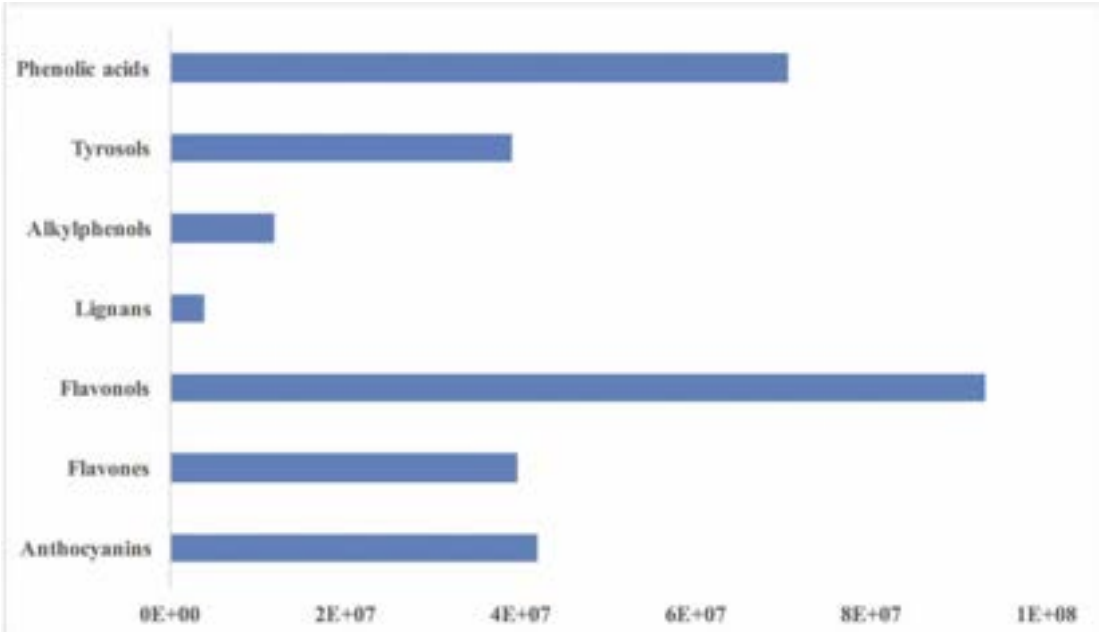

Figure 8: Evaluation of Phenolic Profile by UHPLC-ESI-QTOF-MS: Abundance of different chemical classes of polyphenols in Ficodindia di San Cono PDO cladodes analysed (cumulative intensities as gained from UHPLC-ESI-QTOF-MS profiling).

Flavonoids were definitely the most frequent class of polyphenols with 89 compounds annotated, followed by phenolic acids (54 compounds), tyrosols equivalents detected, with 89 compounds annotated, followed by phenolic acids (54 compounds), tyrosols (27 compounds), and few other phenolics (lignans, alkylphenols and stilbenes derivatives). (41) The main phytochemicals associated to the cladodes and fruits of *Opuntia ficus-indica* include carotenes, ascorbic acid, phytosterols, chlorophyll, betaxanthins, betacyanins, taurine and flavonoids, being the isorhamnetin diglycosides and triglycosides the most abundant. Presence of these isorhamnetin glycosides has been associated with the antiproliferative activity of OFI extracts against HT-29 cells (46) Although the flavonoid isorhamnetin and its glucoside are mentioned as the flavonoid components of the flowers however, penduletin, luteolin, kaempferol, quercetin, quercetin and rutin were isolated and identified. Other reports indicated that the plants of the Cactaceae family contain flavonol 3-O-glycosides (quercetin, kaempferol, and isorhamnetin), dihydroflavonols, flavonones, and flavanonols. (47)

Guevara-Figueroa et al. (48) detected the presence of gallic acid (6.4 to 23.7 μg/g dry weight basis), coumaric acid (140.8 to 161.8 μg/g dry weight basis), 3,4-dihydroxy-benzoic acid (0.6 to 25.1 μg/g dry weight basis), 4-hydroxybenzoic acid (5.0 to 47.2 μg/g dry weight basis), ferulic acid (5.6 to 347.7 μg/g dry weight basis) and salicylic acid (5.8 to 35.4 μg/g dry weight basis) in the prickly pear cladodes and identified iso-quercitrin (22.9 to 396.7 μg/g dry weight basis), isorhamnetin-3-O-glucoside (45.9 to 322.1 μg/g dry weight basis), nicotiflorin (28.9 to 1465.0 μg/g dry weight basis), rutin (23.6 to 261.7 μg/g dry weight basis) and narcissin (146.9 to 1371.0 μg/g dry weight basis) in cladodes of different *Opuntia spp*. varieties.

7.2 Antioxidant effect of OFI

The antioxidant actions attributed to prickly pear fruit can be due to the presence of several compounds, namely vitamin C, carotenoids, but also polyphenols and flavonoid compounds like quercetin, kaempferol and isorhamnetin. Despite some differences within the composition of different cactus structures, it is possible to find some similarities in phytochemicals composition. (45) Boutakiout et al. (49) have suggested that prickly pear cladodes are a good source of natural antioxidant compounds. The authors evaluated antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH•), 2,2-azino- bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and ferric reducing antioxidant power (FRAP) assays of prickly pear cladodes.

The experimental study conducted by Saad et al. (50) has shown that the O. ficus-indica cladode extract (100 mg/kg body weight) was able to reduce the oxidative lithium-induced damage through the increase in antioxidant enzyme levels in the hepatic tissues (superoxide dismutase, catalase, and glutathione peroxidase). This effect is probably associated with the capacity of this extract to reduce the lipid peroxidation level in membrane cells by scavenging free radicals .

Experimental data also suggested a protective effect of ethanolic extract of cladodes found in methotrexate[3]-induced damage in rat intestine (51) and in kidney dysfunction

[3] Methotrexate (MTX) is a cytotoxic chemotherapeutic element for various inflammatory diseases. (51)

via antioxidant, anti-genotoxic and antiapoptotic properties against cis-diamine dichloroplatinum[4]. (52)

Akacha et al. (51) demonstrated that the combined treatment of methotrexate with O. ficus-indica extract significantly contributes to reduce the induced oxidative damage of methotrexate that is a chemotherapeutic element for various inflammatory diseases. The antioxidant actions from the compounds present in *O. ficus-indica* can justify the interest in the prickly pear extracts prepared, using not only the pulp but also cladodes for pharmaceutical use, but also the importance of including the fruit in daily diet. (45)

7.3 From Waste to Wealth

Consumer interest in foods with enhanced nutritional quality has increased in recent years. The nutritional and bioactive characterization of fruits and their by-products, as well as their use in the formulation of new food products, is advisable, contributing to decrease the global concerns related to food waste and food security. Moreover, the compounds present in these raw materials and the study of their biological properties can promote health and help to prevent some chronic diseases. *Opuntia ficus-indica* (L.) Mill. (prickly pear) is a plant that grows wild in the arid and semi-arid regions of the world, being a food source for ones and a potential for others, but not properly valued. (45) Inside its pads there is a mucilaginous substance which represents the plant's water reserve. Many people during years who have started growing prickly pears have wondered if was possible to use this plant and recover its waste, its pads and the waxy wall of the cuticle. (38)

O. ficus-indica is gaining interest across the world because it can grow where no other crops are able to do that. This the case of some countries, such as Ethiopia, where it is

[4] CDDP (cis-dichlorodiammineplatinum (II), CDDP) is a synthetic anticancer drug extensively used clinically for the treatment of several human malignancies such as ovarian, testicular, bladder, head and neck, and uterine cervix carcinomas. Various data indicate that CDDP induces oxidative stress, lipid peroxides and DNA damag. (52)

the only crop that can be relied on. (45) The cactus pads (or cladodes) commonly known as nopales, when cut into small pieces, are regularly consumed as vegetable or forage (16% or 22% of productive lands, respectively) in Mexico, since cactus pear is deeply embedded in local culture. (38) From Mexico the first experiments were made by the scientist Sandra Pascoe Ortizis, a Mexican researcher and chemical engineer (Universidad del Valle de Atemajac). Thus , was born the biodegradable plastic derived from the prickly pear. By processing the prickly pear juice, extracted from the pads of the plant, through natural thickeners, without toxic substances, a 100% biodegradable film is obtained. (53) Starting from these first experiments, a project is now being developed that provides for large-scale industrial production. In this way, an edible, non-toxic, biodegradable bioplastic began to be produced. An organic plastic that does not pollute, a way to make space for the circular economy, starting from plants and drastically reduce the production of plastic.

We have also an example in Italy, Loretta Bacchetta, an ENEA researcher who studies biomaterials in the laboratory of Bioprocesses and Bioproducts (Division of Biotechnology and Agroindustry). She came up with an idea starting from the prickly pear pads. First, in Sicily, producers of prickly pear-based juices and preserves were looking for different ways to use the sticky substance present in the blades of this plant. On the other hand, the need to have a mortar for restoration work on cultural heritage has led them to think of this plant with a thousand uses. In this way, a project was born to obtain a substance from the mucilage of prickly pear pads to be used as a glue. Mucilage itself is a sticky substance: with appropriate additions, it transforms into a good fixative, which is not attacked by fungi or microorganisms and is therefore a very suitable substance for the restoration of cultural heritage.

In addition to its use in the diet or in the restoration, prickly pear is also used for healthcare due to its high content of polyphenols and antioxidant, anti- inflammatory and anxiolytic properties. *O. ficus-indica* is a multipurpose crop, not only to provide food and feed but as a source of bioactive compounds with promoting health properties. (45)

7.4 Opuntia Ficus Indica: an invasive alien species

Alien species are organisms (plants and animals, but also fungi, bacteria and viruses) introduced by humans outside their natural range, either intentionally or accidentally. Some of them successfully establish and thrive in the new environment, having negative impacts and becoming invasive. It is these invasive alien species (IAS) that pose serious threats to the survival of many native species and are among the main causes of biodiversity loss. They also can have serious social, economic and human health consequences.(54)

The cactus (genus *Opuntia*, family Cactaceae) is a native plant of the American continent and is commonly found at every latitude although it is better adapted to arid areas. Prickly pear cacti can be found all over the world, with a wide climatic tolerance, being able to proliferate in rainfall regimes of 250 to 1200 mm per annum with very hot summers of over 40 ◦C, and cold winters with temperatures frequently falling below 0 ◦C for brief durations. Nowadays, there are more than 250 species, distributed in Mediterranean Europe, India, the Middle East and in the American and African countries. (41) As it was said *Opuntia Ficus Indica* is well adapted to arid lands and to diversity of climates all over the world. In the Mediterranean areas, South Africa and South America this species is cultivated for its edible fruits (prickly pear), although in some countries different parts of the plant are utilized in food and cosmetic industry.(38)

Thanks to development the extensive of the root system, it has the capacity to adapt itself to poor and superficial soils, natural or anthropized environments. It has a wide ecological plasticity and tolerates different types of stress such as drought, salinity and fire. The invasions of *O. ficus-indica* have occurred in those countries that have cultivated it for more than 100 years, such as Australia, Eritrea, Ethiopia, South Africa and Hawaii, the United States and also to a certain extent in Somalia and Yemen. In Europe it was introduced around 1500. Man is by far the most important vector for *Opuntia*: in fact, it is the most widespread and commercially most important cactus species, widely introduced for the fruits, and more recently as part of forestry or agroforestry projects in developing countries. The invasiveness is given by the fast

diffusion due to the high seed production and vegetative propagation: unlike the native sites, in the invaded areas there are no natural competitors (parasites) or animals that feed on it, leading to a decrease in the soil for agricultural use. The eradication, cutting and removal as a control method are very laborious and can get worse the infestations producing new cuttings. For this reason the pruning residues must be incinerated, avoiding composting. (55)

Figure 9: Invasion of *Opuntia ficus-indica* in Punta dell'Arco, Ventotene.

AIM OF THE THESIS

The aim of the present master thesis work is to characterize the possible role of *Opuntia Ficus Indica*'s waste material (namely, the cladodes) in the field of cancer research.

The interest in the nutritional and bioactive characterization of fruits and their by-products, as well as their use in different formulation, has increased in recent years due to their potential health-related functions, including antioxidant, anti-inflammatory, anti-proliferative and anti-microbial properties for both disease prevention and therapeutic options. Despite many preliminary clinical and experimental evidences, the study of their biological functions and applications is a still on-going and promising research field for both scientific and economic reasons.

Opuntia ficus-indica (L.) Mill. (OFI) (prickly pear) is a plant that grows wild in the arid and semi-arid regions of the world, being a rich food source in many countries worldwide. Once the fruits have been collected, a huge amount of waste materials remains from plants' cultivation, representing a significant cost for producers who do not practice the so-called "productive pruning", that is the use of waste for new production cycles, in accordance with the European principles of bioeconomy and circular economy. In this context, the reduction and possible reuse of wastes discarded by the food industry has become a master topic of the European Commission that is strongly supporting several actions against food waste.

The Directive 2008/98/EC [5] on waste (Waste Framework Directive) indicated the criteria for defining a waste as a secondary raw material, which has its own cycle industrial and can follow a diversified use chain to obtain new primary products or different useful stuff.

According to these criteria, the thesis work was aimed at testing the possible use of waste cladodes from OFI for application in the experimental cancer research field. OFI is rich in polyphenols, a class of biomolecules known as micronutrients that have been increasingly proposed as valuable bioactive molecules for promising applications in

[5] This Directive establishes measures to protect the environment and human health by preventing or reducing the negative impacts of waste generation and management, reducing the overall impacts of resource use and improving their effectiveness.

the prevention of various diseases, such as diabetes, cardiovascular diseases, neurodegenerative diseases, liver disease, and cancers.

For research purposes, I used the *Opuntia Ficus Indica*'s fresh gel (extracted from the cladodes and characterized at the ENEA Research Lab in Rome) and tested its potential anticancer effect in the Caco2 human adenocarcinoma cell line.

Based on the literature's evidence, I focused on the antioxidant ability of the matrix, in particular on S-nitrosoglutathione reductase (GSNOR)-related antioxidant pathways.

MATERIALS AND METHODS

1. PLANT MATERIALS

The OFI's gels used in this experimental thesis were a gift from the Department of Bioprocesses and Bioproducts (ENEA Casaccia, Division Biotecnology and Agroindustry, Rome, Italy).

The cladodes were obtained from O. ficus-indica cv. *Sanguigna plants* grown in the region of Maccarese, Rome. The cladodes were collected early in the morning, from the beginning of June through October. The 2–3 years old cladodes were immediately used for the experimental trials. These were washed with tap water in order to remove impurities and spines, peeled from epidermis in both sides and then chopped into 1–2 cm pieces. (38)

1.1 EXTRACTION CONDITIONS

Biomass-to-water ratio (w/v), pH, ionic strength, temperature

The colleagues of Department of Bioprocesses and Bioproducts (ENEA Casaccia, Division Biotecnology and Agroindustry, Rome, Italy) defined five biomass-to-water ratios: 1:1, 1:3, 1:5, 1:7, 1:9 (w/v); 200 g of fresh chopped cladodes. They were put in flasks with distilled water (200 mL in 1:1, 600 mL in 1:3, 1000 mL in 1:5, 1400 mL in 1:7 and 1800 mL in 1:9), then they left them soaking for 24 h at 25 °C, in dark condition without stirring. They filtered all the samples with a fine cloth (0.5 mm) until there was no mucilage dropping down. The mucilage was maintained at - 5 °C until the analysis. Each experiment was replicated three times.

Based on literature (56) they investigated a range of pH from 2 to 12 with and without buffer as a control. Fifty grams of fresh chopped cladodes in 250 mL of distilled water at pH 2.0, 4.5, 7.0, 9.5, 12.0 with and without a buffer (biomass-to-water ratio was 1:5) were replicated three times each. They used either hydrochloric acid or sodium hydroxide to adjust the pH in the samples without buffer and the McIlvaine's tables (Sigma-Aldrich) to prepare the samples with buffer. The pH value was determined before and after 24 h of soaking. In order to evaluate the effect of ionic strength, 50 g of fresh chopped cladodes were added to 250 mL of distilled water (biomass/water

1:5) supplemented with NaCl or $CaCl_2$ at the concentration of 0.1, 1.0, 10.0 and 100.0 mM. A total of eight samples were compared to the control sample (50 g of plant material in 250 mL distilled water) after 24 h of soaking. Each experiment was carried out in triplicate. Mucilage was extracted from 50 g of fresh chopped cladodes in 250 mL of distilled water for 24 h at 20 or 80 °C. All solvents were purchased from Sigma–Aldrich (Milan, Italy). (38)

Ultrasonic and microwave assisted extraction of mucilage

In order to increase the mucilage recovery, two sets of three times replicated samples (50 g of fresh chopped cladodes in 250 mL of distilled water, 1:5 w/v), have been subject to: (1) ultrasonic bathing (EMAG ZCC009, United States) at 40 kHz for 30 min; (2) microwaves (SMEG ME/ 203FX, Italy) at 500 W for 5 min. The percentage muci- lage yield extracted from each samples was compared to the extraction obtained by conventional method (50 g of fresh chopped cladodes in 250 mL of distilled water at room temperature in the dark). Data were recorded a t0 (immediately after treatment); at t1 (after 16 h of maceration); at t2 (after 24 h of treatment). (38)

1.2 CONTENT ANALISYS

The total content of OFI gels and polyphenols was determined according to the colorimetric method of Folin-Ciocalteu with some modifications and the results were expressed in mg eq of gallic acid by interpolation of the calibration line Abs:750 vs gallic acid mg /ml.

The antioxidant activity of polyphenols was determined through the comparison with the radical DPPH, the calibration line (Abs) vs % of inhibition was prepared using, as the reference reducing agent, the Trolox, a synthetic water-soluble analogue of vitamin E. The percentage of inhibition, obtained by interpolation, is expressed by this relationship = 1- (Ac / A0) x 100, where Ac is the absorbance of the sample and A0 of the blank. (44)

$$DPPH\ radical\ scavenging\ activity = \frac{Abs\ control - Abs\ sample}{Abs\ control} \times 100$$

The polyphenol content and antioxidant activity are significantly important. As already mentioned, few data are available in the literature on the phenolic component and on the antioxidant power of the mucilage of *Opuntia Ficus-indica*. The analyzes carried out in our laboratories show an average total polyphenol content of 14.2 ± 0.03 mg GAE g-1 dry weight with an antioxidant capacity of 6.8 ± 0.5 μmol TE g-1 dry weight.

It was also performed a qualitative test with HPLC[6] (Figure 10) on the following polyphenolic compounds: acid p-coumaric, quercetin, rutin, hyperoside, iso-quercetin, kaempfe-rol-3-O-glucoside and Gallic acid. The different molecules were identified by comparing them with standards, through retention time and UV spectra. (44)

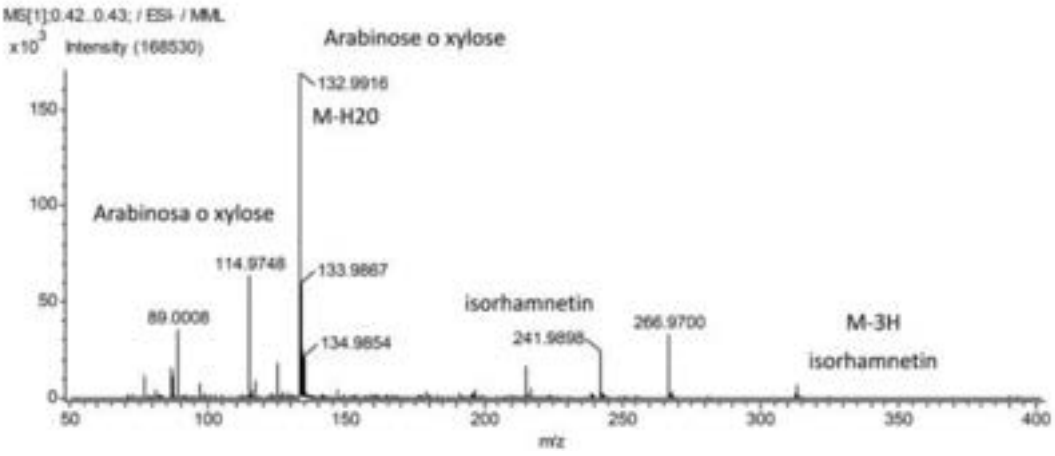

Figure 10: DART_MS spectrum (at 300 ° C in negative mode) of the mucilage

[6] Agilent Infinity 1260 equipped with Supelco LC8 column, quaternary pump, automatic injector mounted on the autosampler module and DAD detector.

2. CELLULAR BIOLOGY

2.1 CELL COLTURES: CACO2 CELLS

For this study, Caco-2 cells were used to perform all the experiment. Caco-2 are epithelial cells isolated from colon tissue derived from a 72-year-old, white, male with colorectal adenocarcinoma. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with GlutaMAX, 10% fetal bovine serum (FBS) and 1% of penicillin/streptomycin (100 U/mL), in the incubator with 5% of CO_2 and 37°C of temperature. All media and supplements were purchased from Thermo Fisher. Caco-2 cell line grows very fast. In Figure 11 and 12 is it possible to appreciate their confluence after 24h and 56h post seeding.

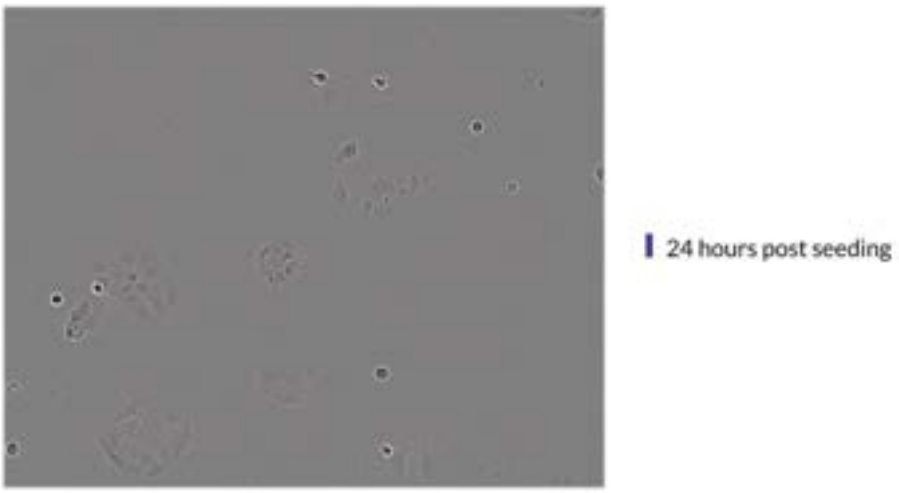

Figure 11: Caco-2 cells after 24h of confluence

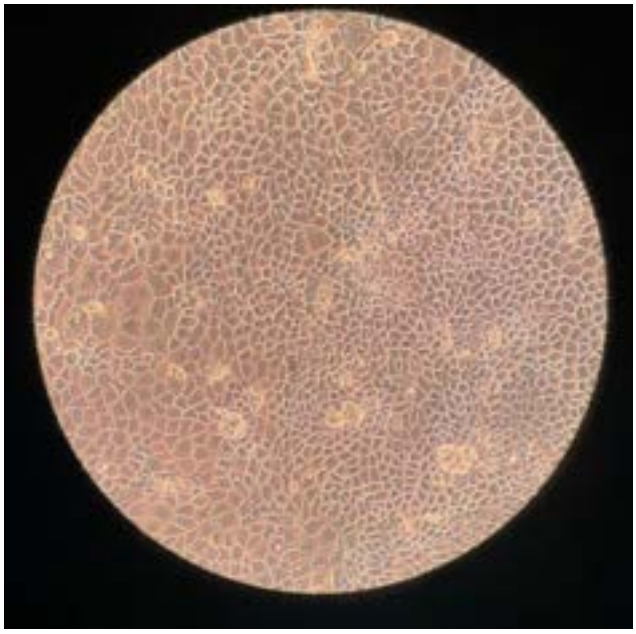

Figure 12: Caco-2 cells after 56h of confluence

2.2 MONOLAYER CULTURES: CHARACTERISTICS AND PROPERTIES

The Caco-2 cells spontaneously differentiate to express morphological (polarized columnar epithelium) and functional characteristics of mature small intestinal enterocytes, so called monolayer. Monolayer of Caco-2 were obtained by cultivating the cells for 21 days of confluence. The medium of cells was replaced every three days.

2.3 TREATMENTS

2.3.1 Opuntia Ficus treatment

From the extraction processes, two different gel extracts of *Opuntia Ficus Indica* were obtained: one from fresh gel and other from flour gel. Different concentration of both the extracts were used to treat the cells. The *Opuntia Ficus Indica*'s fresh gel was used in a range of 0 to 5000ug/mL, instead the *Opuntia Ficus Indica*'s flour gel was used in a range of 0 to 50 ug/mL. Caco-2 cells were seeded at $0.8x10^{\wedge 5}$ cells/mL and

incubated for 24-56h with OFI's extract. At the end of treatments, cells were analyzed for the specific assay.

2.3.2 Proteosome and autophagy machinery inhibition

Inhibition of proteasome and of autophagy function was done by treating Caco-2 cells with MG-132 10 uM for 5 hours and Bafilomycin 50 nM for 5 hours, in the presence or absence of OFI.

MG132 is a potent, reversible, and cell-permeable proteasome inhibitor. Bafilomycin is a a potent inhibitor of cellular autophagy and it specifically targets the vacuolar-type H^+-ATPase (V-ATPase) enzyme, a membrane-spanning proton pump that acidifies either the extracellular environment or intracellular organelles such as the lysosome.

3. IN VITRO ANALYSIS

3.1 VIABILITY ASSAY

Caco-2 cells were seeded in 24-wells plates at the concentration of $0.8x10^{\wedge 5}$ cells/mL or $1x10^{\wedge 5}$ cells/mL and treated with different concentration of OFI (see section *Opuntia Ficus Indica* treatment). After 24h and 48h of treatment, medium was removed and replaced with Alamar Blue solution (Resazurin sodium salt 56 μM in full medium, Sigma-Aldrich) and viability was assessed. AlamarBlue Cell Viability Assay reagent quantitatively measures the proliferation of cells. The dye incorporates an oxidation-reduction (REDOX) indicator that both fluoresces and change colour in response to the chemical reduction of growth medium due to cell growth. In order to let the dye incorporation, cells were incubated for 1-2 h at 37 C. After the incubation time, 100 uL of each condition was transferred into a 96-wells black plate. The emission fluorescence was recorded at 590 nm and upon excitation at 530-560 nm by a microplate reader iD3 SpectraMax (Molecual Devices). Relative fluorescence of each condition was calculated as fold change with respect to the untreated cells (control, Ctr) after baseline fluorescence (blank) subtraction (Alamar Blue solution incubated in a cells-free sample).

4. PROTEINS BIOCHEMISTRY

4.1 Cell collection and protein lysates preparation

Working on ice, the medium of cells was removed, and cells were washed twice with Dulbecco's Phosphate Buffered Saline (DPBS, ThermoFisher) in order to remove any medium residues. Then lysis Buffer (Tris HCl 50mM pH 6.2, SDS 2%, Glycerol 10%) was added directly on plate and cells were incubate for 10 min at room temperature (RT). After incubation time, cells were mechanically detached from the plate by a scraper and collected in microcentrifuge tubes. Samples were then boiled at 95°C for 10 min in agitation. Then, samples were frozen at -20°C for long storage or directly used.

4.2 PROTEIN QUANTIFICATION

Protein quantification of cell lysates was performed by using the DC Protein Assay (Bio-Rad), based on Lowry method. The Lowry protein assay is a biochemical assay for determining the total levels of proteins in a solution. The total proteins concentration is exhibited by a colour change of the sample solution in proportion to proteins concentration, which can then be measured using colorimetric techniques.
The method involves the use of two reagents A (25uL) and B (200uL) and the quantification of the protein content using a multiplate reader (Victor Spectrofotomer) equipped with a filter to measure absorbance at l = 750 nm. The value of the proteins contained in each sample is obtained by interpolating the absorbance on a standard reference line (standard curve) constructed with defined and increasing concentrations (2,4,8,16 ug/mL) of Bovine Serum Albumin (BSA).

4.3 ELETTROFORESIS GEL (SDS-PAGE)

SDS-PAGE (Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis) is commonly used as a method to separate proteins with molecular masses between 5 and 250 kDa. The combined use of sodium dodecyl sulfate (SDS, also known as sodium

lauryl sulfate) and polyacrylamide gel allows to eliminate the influence of structure and charge, and proteins separation is based only on their molecular weight.

After the quantification, samples were denatured with sample buffer (v/v 40% glycerol, v/v 20% Tris HCl 1M pH 6.8, w/v 8% SDS, w/v 0,1% bromophenol Blue , v/v 28% H2O, 4% Reducing agent ; Thermo Fisher Scientific) at 95 ° C for 3 min in agitation.

Thus, 15-30 μg of proteins were directly used and loaded into the gel in the presence of a molecular weight marker from 10 kDa to 250 kDa (PageRulerTM Plus - Thermo Fisher Scientific). The separation of proteins was possible by the presence of a special running buffer (Tris-Glycine, SDS 1%) and by the application of a constant voltage of 120 V.

4.4 WESTERN BLOT

Western blotting is a well-established analytical technique for detecting, analyzing, and quantifying proteins. This method is widely used to detect specific protein molecules in complex samples such as cell lysates. Western blotting typically involves protein separation by gel electrophoresis followed by transfer to a polyvinylidene difluoride (PVDF) or nitrocellulose membrane.

A range of 15-30 μg of protein extracts were electrophoresed by SDS–PAGE (18-26 wells 4-15% stainfree gel) and blotted onto PVDF membrane. After the running, proteins have been transferred by a semi-dry system using the Trans-bot Turbo RTA Midi 0.2 um PVDF Transfer Kit (Bio-Rad).

In order for the transfer to take place, there is a need to activate the PVDF membrane in two steps with two plastic containers. This involves passing the membrane into pure ethanol for 30 seconds and into transfer buffer (Tris HCl 25 mM, Glycine 190 mM, ethanol 20%) for other 30 seconds and place a sponge stack in the trans-blot cassette, followed by the membrane, the gel and lastly another sponge stack, rolling out all the bubbles.

After the “2 mini or 1 midi gel” 30 minutes transfer program, a-specific sites were saturated by incubating the PVDF membrane with non-fat milk 5% dissolved PBS-Tween at 0.01% (T-PBS), for 1 h at room temperature in gentle agitation.

For the detection of the proteins of interest, the membrane was cut in different fragments and each part incubated with primary antibody for 1-3 h at room temperature, or overnight at 4 °C (see Table 2 for the list of antibodies used). After incubation time, primary antibodies were removed, and membrane was washed thrice with T-PBS before adding the secondary antibodies (Goat Anti-Rabbit (BIO-RAD®), o Goat Anti-Mouse (BIO-RAD®)) for 1 h at room temperature in agitation. Secondary antibodies were removed, and membrane was washed thrice with T-PBS before the image acquisition. The primary and secondary antibodies have been dissolved, with a dilution ranging from 1: 1000 to 1:3000, in a solution of 5% non-fat milk and T-PBS. Image acquisitions were visualized and taken by ChemiDoc MP Imaging System (Bio-Rad), upon addition of chemiluminescence reagent solution (ECL Prime Western Blotting Detection Reagent, Amersham). After incubation with the ECL detection system, images were adjusted for brightness and contrast. Densitometric analysis consists in the study of quantitative and qualitative patterns of distributions of intensity maps of gray levels of image components to examine.

"ImageLab" is the software of the Bio-Rad company for imaging, processing and analysis of gels and blots using the ChemiDoc MP tool, which can be used both in a Windows environment both in the Macintosh. For western blotting, endogenous Vinculin was used as an internal reference and the results of the densitometric analysis were reported as arbitrary densitometric units normalized to Vinculin.

Table 2. Antibodies

Name of Primary Antibodies	**Name of Secondary Antibodies**
Anti-GSNOR	Anti-Mouse
Anti-AKR1A1	Anti-Mouse
Anti-MgSOD	Anti-Rabbit
Anti-Vinculin	Anti-Mouse

5. MOLECULAR BIOLOGY

5.1 RNA EXTRACTION

RNA extraction was done by using the NucleoSpin® RNA isolation kit (Machery-Nagel) and protocol. Briefly, Caco-2 cells pellet was lysated, filtrated and cleared by filtration through NucleoSpin® Filter. After purification, to remove DNA contamination, 95 µL of DNase reaction mixture was added, and incubated at room temperature for 15 min. Finally, washing steps were done and total RNA was solubilized in 40-60 µl of RNase-free water. The concentrations and purity of RNA was quantified using a Nanodrop spectrophotometer at absorbance values of 260 nm. The purity of the RNA extracted were determinate by analyzing the absorbance ratio A260/280 in which RNA is pure if this ratio is between 1.8 and 2.0.

5.2 REVERSE TRANSCRIPTION

Reverse transcription is a process in which copy DNA (cDNA) is synthesized by the reverse transcriptase enzyme from an RNA preparation. The cDNA can be used as a starting material for PCR. Through this process they are produced libraries of cDNA that represent only those genes that are transcribed and expressed in the used tissue/cell culture. The reverse transcription reaction was performed using a commercial kit (Promega, Wisconsin) which required the preparation of two solutions.

Solution 1:

- RNA, 0.5-1 µg of total RNA diluted with double distilled water and brought to the same concentration.
- Oligodt (200 nM), an oligonucleotide of about 20 base pairs long containing only thymine that pairs to the poly-A tail present in mRNA molecules, thereby enriching the cDNA library of protein-coding RNAs.
- Random primers (200 nM), a mixture of random sequence oligonucleotide fragments that generates a pool of cDNA of different lengths.

The samples were placed in the thermal cycler for 5 minutes at 70 ° C to induce denaturation by breaking the hydrogen bonds that determine the secondary structures of RNA. At the end of this operation the samples were left on ice.

Solution 2:

- Buffer 5x and $MgCl_2$ (final concentration 5 mM), a mixture of salts optimized for the correct functioning of the reverse transcriptase.
- dNTP (final concentration 0.5 mM for each), an equimolar mixture of triphosphate deoxynucleotides used in the polymerization process by reverse transcriptase.
- RNase inhibitor (20 U), a pharmacological inhibitor of enzymes with RNase activity.
- Double-distilled water (ddH_20), necessary to bring the solution to volume
- Reverse transcriptase (RT) (200 U / μg of RNA)

The two solutions were mixed, inserted in the thermal cycler by setting the following cycle: 1h at 40 ° C and 15 min at 70 ° C (temperature necessary for the final denaturation of the enzyme). The produced cDNA was immediately processed or, alternatively, stored at a temperature of -20 ° C.

5.3 QUANTITATIVE PCR (REAL-TIME PCR)

PCR (Polymerase Chain Reaction) is a technique based on the amplification of DNA or cDNA fragments using a specific sequence of oligonucleotides. In traditional PCR, the analyzes are performed by agarose gel electrophoresis at the end of the last PCR cycle and provide qualitative data. Real-time PCR (RT-qPCR) is a type of semi-quantitative PCR in which the amplification products can be measured during the polymerization process (Kubista M. et al., 2006). The instantaneous detection of the amplification process is based on the use of fluorescent probes such as SYBR green, a fluorophore that intercalates in the double-stranded DNA emitting fluorescence. The SYBR green fluorescence increases during amplification in proportion to the accumulation of the double-stranded amplifier. The peculiarity of real-time PCR lies in the fact that the increase in fluorescence is directly proportional to the number of amplified molecules. By monitoring the reactions during the exponential phase, the initial quantities, relative to a control, of the target sequence can be determined with great precision.

RT-qPCR was performed using the iTAQ universal SYBR Green Supermix (Bio-Rad Laboratories) on a ViA 7 Real-Time PCR System (Applied Biosystems). Data were analyzed by the ViA" 7 Software using the second-derivative maximum method. The fold changes in mRNA levels were determined relative to a control after normalizing to the internal standard L34.

The amplification mix for each gene was prepared into classic 1.5-2 mL eppendorf and contain: cDNA, the DNA pol. Enzime Green Supermix 1000 x 20uL rxns, ddH_2O Rnasi/Dnasi free up to volume, 8 uL FW primers 1uM and 8 uL RV primers 1uM and dNTPs and 10uL SYBER Green 2X. In order to hybridize the regions of the appropriate gene sequence of interest, specific sets of primer pairs were used.

Reaction was performed according to the protocol below.

Table 3. qRT-PCR amplification cycle program

Reaction Phase	**Temperature**	**Timing**
Initial Denaturation	95°C	5 min
Denaturation	95°C	13 sec (n. 40 cycles)
Annealing / Elongation	60°C	40 sec

Table 4. All genes

Acronym	Name of the gene	Initial Concentration
GSNOR	S-nitrosoglutathione reductase	50uM
HMOX1	heme oxygenase-1	100uM
GCL	Glutamate–cysteine ligase,	100uM
NQO1	NAD(P)H:quinoneo xidoreductase 1	100uM
L34	Homo sapiens ribosomal protein	50uM

6. STATISTICAL ANALYSIS

Both the production of the graphs and the statistical analysis of the data were carried out using the GraphPad Prism software and for the protocols it was used Image Lab. The graphs show the data as histograms representing the mean ± s.e.m (standard error of the mean). The statistical analysis was carried out with the ANOVA (Analysis of Variance) and Student's t test one-way statistical tests. The data with p-value <0.05 were considered significant, where p is the level of significance observed. Precisely we have indicated with *, values of p <0.05; **, p-values <0.005; ***, p values <0.001; ****. We considered p values> 0.05 to be insignificant (n.s.)

RESULTS

1. Opuntia Ficus Indica's CONTENT ANALISYS

The analysis performed by ENEA lab shown that *Opuntia Ficus Indica*'s gel is a multi-functional and very rich matrix.
In particular, the content analysis, shown that the macro-elements with a higher relative content are potassium (28.06% ± 0.52), calcium (3.03% ± 0.06) and magnesium (2.04% ± 0.11). Moreover, results shown that OFI matrix have a significant polyphenol content and antioxidant activity (Table 5).

Table 5. Content in polysaccharides, Uronic acids, Proteins, Antioxidant activity and Polyphenols in the mucilage of Opuntia

Chemical Composition of OFI's mucilage	
Content of Polysaccharides (mg eq xylose g^{-1} dry weight ± ES)	33.9 ± 2.49
Content of Uronic Acids (mg uronic acids g^{-1} dry weight ± ES)	1.15 ± 0.04
Content of Proteins (mg eq ABS g^{-1} dry weight ± ES)	37.93 ± 0.02
Antioxidant Activity (μmoli TE g^{-1} dry weight ± DS)	6.8 ± 0.5
Total Polyphenols (mg GAE g^{-1} dry weight ± DS)	14.2 ± 0.03

As already mentioned, few data are available in the literature on the phenolic component and on the antioxidant power of the mucilage of *Opuntia ficus-indica*. The analyzes carried out in our laboratories show an average total polyphenol content of 14.2 ± 0.03 mg GAE g-1 dry weight with an antioxidant capacity of 6.8 ± 0.5 μmol TE g-1 dry weight.

Differences with the literature data are attributable not only to different extraction methods but also to the climatic conditions in which the collected material is found. In

any case, the antioxidant power of Opuntia's mucilage is clearly superior when compared with that of apple, banana and pineapple extracts. (57)

2. Analysis of Opuntia Ficus Indica effect on NRF2 pathway

Results above and experiments performed by our colleagues at the ENEA lab have shown an antioxidant activity of OFI gels. Antioxidant properties could be the results of two mechanisms: the pool of biomolecules in the gel matrix exerts antioxidant properties themselves or, alternately, acts as pro-oxidant which induces the activation of antioxidant pathways as a response. In order to investigate these two hypotheses, we evaluated the activation of the master regulator of antioxidant response, the transcriptional factor NRF2. NRF2 is a fundamental in controlling multiple cytoprotective responses through the induction of a complex transcriptional program that ultimately makes cancer cells resistant to oxidative, metabolic and therapeutic stress. (58)

Results shown in Figure 13 indicate that OFI treatments do not affect the activation of NRF2 pathway. qPCR analysis, indeed, shows no modulation on the mRNA levels of NRF2-target genes, such as heme-oxygenase 1 (hMOX1), NADH:quinone oxidoreductase 1 (NQO1) and glutamate:cysteine ligase (GCL) assayed in cells incubated with different OFI concentrations for 6, 12 and 24 h. This result suggests that the pool of biomolecules contained in OFI gels exerts antioxidant properties themselves.

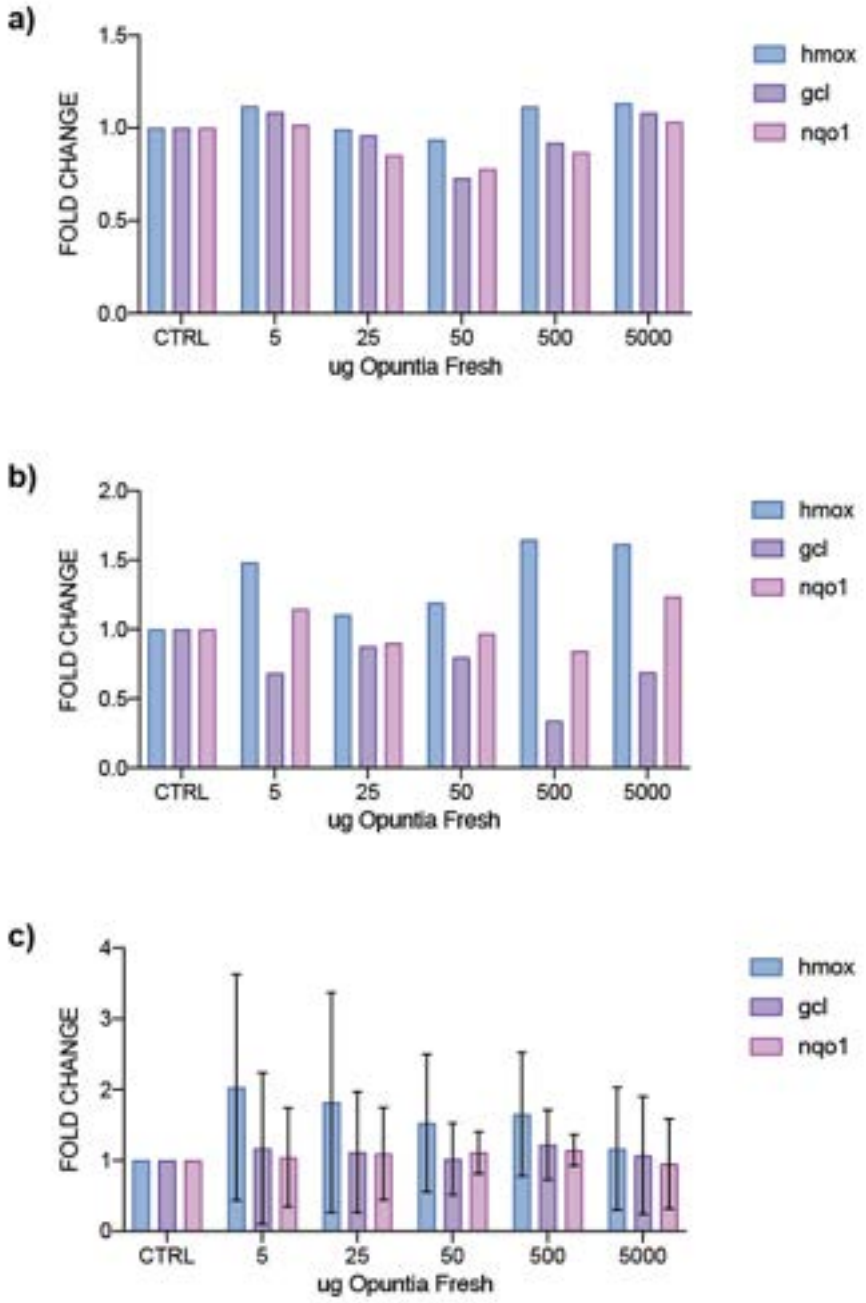

Figure 13: Dose response and time dependence analyses of NRF2-responsive gene expression upon incubation with Opuntia Ficus Indica's fresh gel extract. Expression of hmox, gcl, nqo1 were assesses by qRT-PCR in CaCo2 cells incubated with different doses (from 5 to 5000 µg) of Opuntia Ficus Indica's fresh gel extract treatment for 6h (**a**), 12 h (**b**) and 24 h (**c**) of incubation. Only the experiment at 24 h was performed in triplicate.

3. Analysis of Opuntia Ficus Indica effect on cell viability

Evidence from the literature has linked food consumption to the risk of colorectal cancer. However, other observations suggest that a combination of phytochemicals, nutraceuticals, diet, and exercise could prevent the risk of disease. The use of phytochemicals, such as polyphenols, has been proposed as a promising approach for cancer prevention. As mentioned above, analyses conducted by colleagues from ENEA lab showed high levels of polyphenols in OFI matrix, thereby opening new questions on the biological function of this innovative matrigel. In order to understand whether OFI gels might affect cell proliferation and viability, we incubated Caco2 tumor cells with increasing doses of different OFI gel preparations, i.e., flour (dehydrated) or fresh, and evaluated any changes in proliferative ability. Results shown below clearly indicate that the viability of Caco2 cells was not affected by OFI flour (Figure 14), neither when it was applied as fresh preparation (Figure 15).

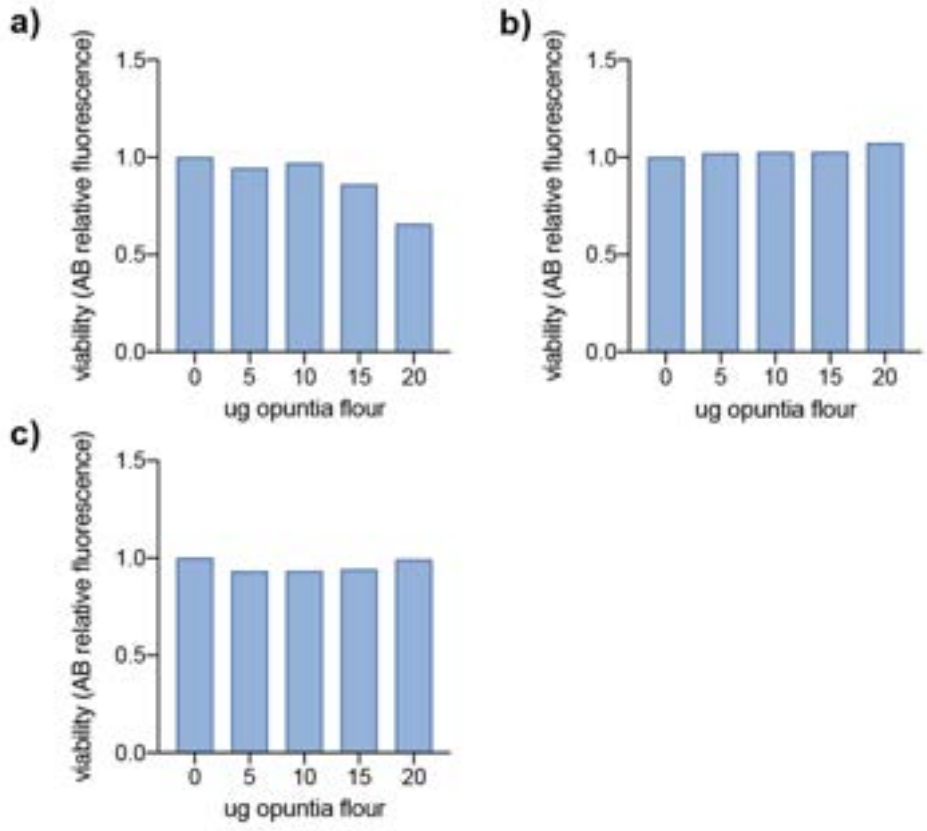

Figure 14: Effect of OFI flour on Caco2 viability. CaCo2 cells were plated and incubated for 24 h (**a**), 48 h (**b**), or 56 h (**c**) with increasing concentrations of Opuntia Ficus Indica fresh gel extracted from cladodes (Experiments were performed in technical but not experimental triplicate).

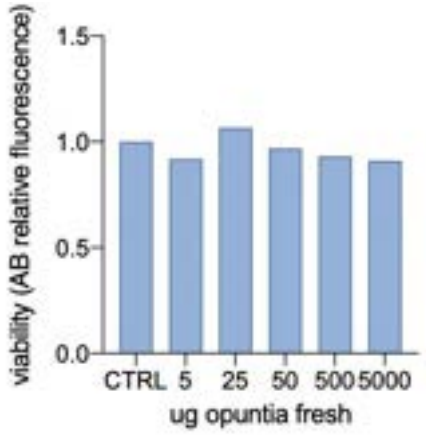

Figure 15: Effect of OFI fresh gel on Caco2 viability. CaCo2 cells were plated and incubated for 24 h with increasing concentrations of Opuntia Ficus Indica fresh gel extracted from cladodes (Experiments were performed in technical but not experimental triplicate)

4. Analysis of Opuntia Ficus Indica effect on GSNOR expression

a. GSNOR expression decreases after OFI treatment

Many lines of evidence suggest that polyphenols can modulate, directly or indirectly, cell signaling and PTMs (59–61) such as *S*-nitrosylation (23,62). Based on this, we wondered if OFI matrix could control NO signaling and so the levels of *S*-nitrosylated proteins. To investigate this aspect, we focused on the denitrosylase GSNOR, which represents the tuner of S-nitrosylation in cells. Indeed, no studies has so far shown the efficacy of OFI treatment on GSNOR expression.

CaCo2 cells were then incubated with OFI extracts, collected at different times and GSNOR levels assessed by Western blot.

Results shown in Figure 16 indicate that GSNOR decreases after OFI treatment in a dose- and time-dependent manner, suggesting an effect of *Opuntia Ficus Indica* on NO signaling.

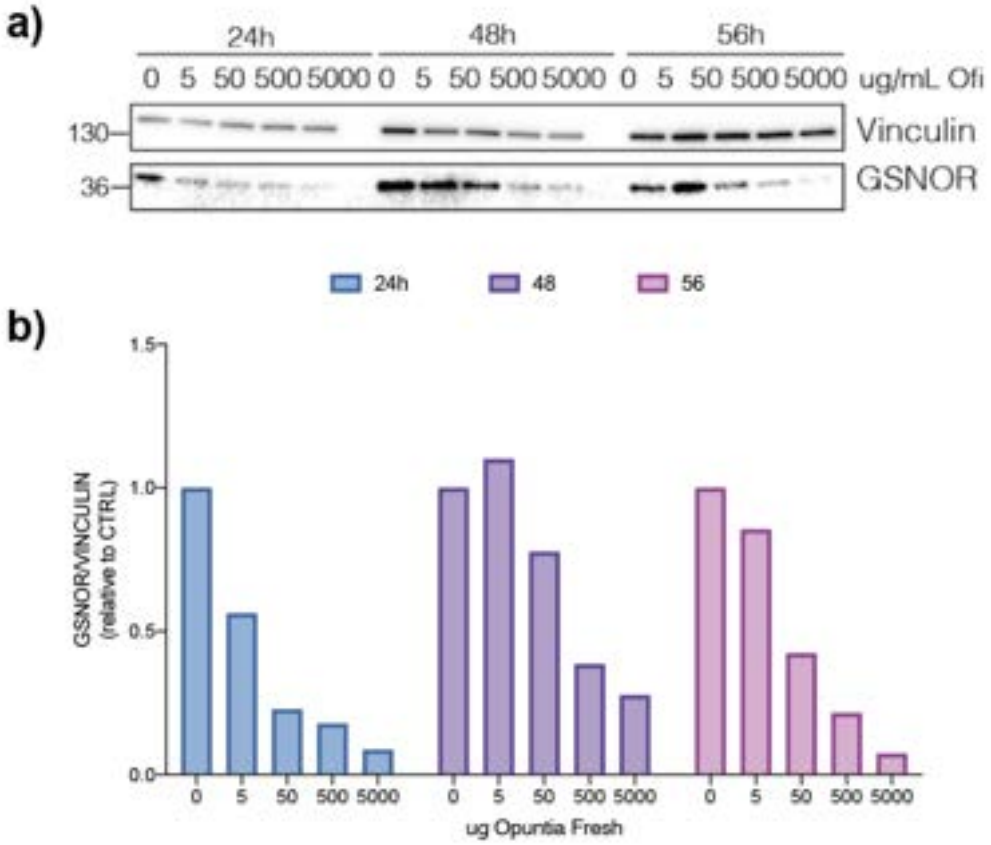

Figure 16: Effects of OFI extract on GSNOR expression. CaCo2 cells were plated and incubated for 24, 48 or 56 h with increasing concentrations (from 5 to 5000 µg) of Opuntia Ficus Indica fresh gel extracted from cladodes. Then cells were lysed and GSNOR levels evaluated by Western blot. Vinculin was chosen as loading control.

In Figure 17, we show the densitometry of GSNOR immunoreactive bands relativized by vinculin as means ± standard deviations of n = 3 experiments performed after 24 h (a) and 48 h (b) of incubation with OFI extracts, which suggest a significant decrease of GSNOR, ranging from 25 to 50% only after 48 h.

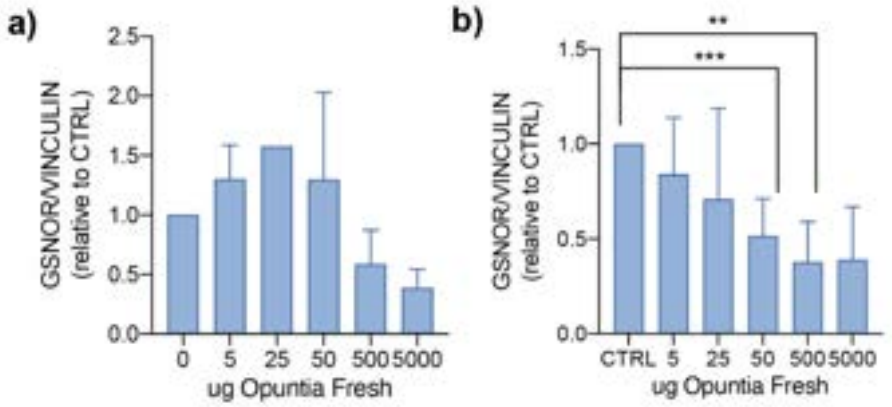

Figure 17: Densitometry of GSNOR immunoreactive bands, CaCo2 cells were plated and incubated for 24 h (**a**) 48 h (**b**) with increasing concentrations of Opuntia Ficus Indica fresh gel extracted from cladodes. Then cells were lysed and GSNOR levels evaluated by Western blot. Densitometry of Western blots from three different experiments were analyzed by FiJi software and normalized on Vinculin (loading control). Statistical significance was calculated by t-Test. $^{**}p < 0.01$; $^{***}p < 0.001$

b. The decrease of GSNOR after OFI treatment does not occurs at the transcriptional level.

As described above, OFI treatment induces a decrease of GSNOR protein levels, but did not know the mechanism involved in this phenomenon. GSNOR decrease could, indeed, be the result of a reduced transcription, or of an accelerated protein degradation. In order to which, of the two proposed mechanisms, was the one underlying the phenomenon observed, we analysed GSNOR mRNA after OFI treatment.

Results shown in Figure 18 indicate that GSNOR mRNA was not modulated by incubations with OFI extracts, suggesting that OFI effect on GSNOR does not occur at the transcriptional level, but probably affects protein stability.

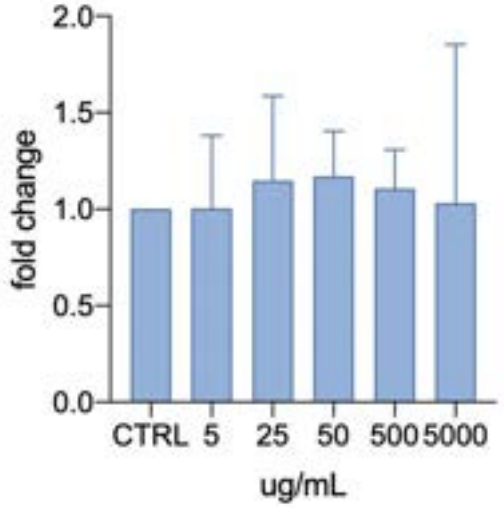

Figure 18: Effects of OFI extract on GSNOR mRNA. CaCo2 cells were plated and incubated for 24 h with increasing concentrations (from 5 to 5000 μg) of Opuntia Ficus Indica fresh gel extracted from cladodes. Then mRNA was extracted and GSNOR mRNA evaluated by qRT-PCR.

c. Autophagy modulates GSNOR protein levels upon OFI treatment.

Results shown so far indicate that GSNOR decrease does not occur transcriptionally, suggesting that OFI gels affected protein stability and as a result, accelerated the rate of GSNOR degradation. Mammalian cells have two different mechanisms to degrade proteins: via autophagy or the proteosome (62). In order to understand, which mechanism was involved in GSNOR decrease, Caco2 cells were treated for 24 h with different concentrations of OFI gel in combination with the inhibitors of autophagy or proteosome: Bafilomycin or MG132, respectively. Then cells were lysed and GSNOR evaluated by Western blot analyses.

As shown in Figure 19, only bafilomycin treatment was able to restore GSNOR protein levels, whereas MG132 seemed to be ineffective, suggesting that autophagy is the only mechanism involved in the modulation of GSNOR protein levels.

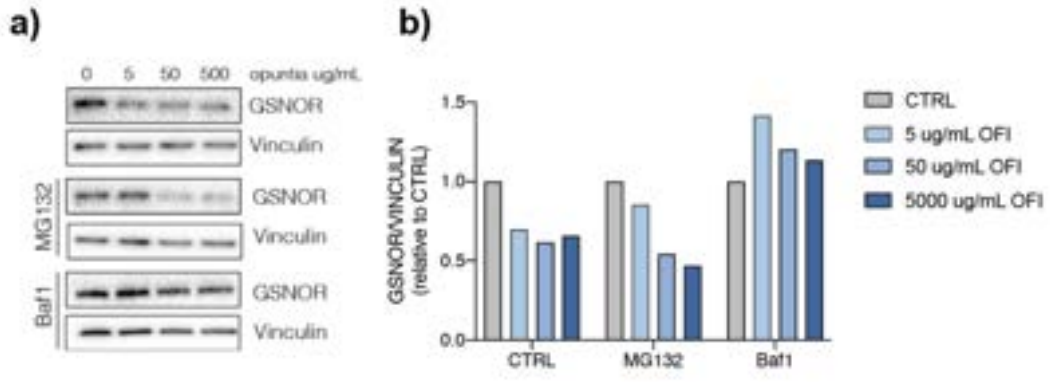

Figure 19: Role of autophagy in the regulation of GSNOR levels by OFI extracts. (a) CaCo2 cells were plated and incubated for 24 h with increasing concentrations of Opuntia Ficus Indica fresh gel extracted from cladodes in combination with the proteasome inhibitor (MG132) or the autophagy inhibitor bafilomycin 1 (Baf 1). Then cells were lysed and GSNOR levels evaluated by Western blot. Vinculin was chosen as loading control. **(b)** densitometry of one representative experiments

5. Analysis of Opuntia Ficus Indica effect on monolayers

Different lines of evidence suggest that polyphenols derived from plants, can exert anti-inflammatory effect and improve cancer prevention (51) (52) (54). Moreover, our collaborator at the Laboratory Health and Environment, ENEA Centro Ricerche Casaccia, demonstrated an anti-inflammatory effect of OFI gel on cancer cell (data not shown). These evidence, together with data obtained in our study, prompted us to postulate that the pool of (still not fully identified) molecules contained in OFI gel might synergistically exert anticancer effect. To investigate this hypothesis, we tried to recapitulate a more physiological condition of OFI absorption. To do this, we took advantage from the property of CaCo2 cells to form adherent monolayers. These monolayers are a good model to simulate drugs absorption and the analysis of them are morphologically and structurally comparable to a biopsy performed on the gastrointestinal tract. Therefore, with the aim to mimic a more physiological epithelial barrier model of human gut, we cultured adherent monolayers of Caco2 cells for 21 days.

Then, to induce epithelium barrier dysfunction in vitro, we applied lipopolysaccharide (LPS). LPS has been reported to induce inflammatory response and epithelium barrier

dysfunction in vitro (64). Moreover, LPS is commonly used to induce in vitro intestinal barrier disease, such as the Inflammatory bowel disease-like.

After the treatment with a combination of OFI gel and LPS, we tested: i) inflammatory response and ii) GSNOR levels. Unfortunately, macroscopically observations suggest that we were not able to induce an appropriate inflammatory response, indicating that our experimental conditions (i.e., LPS concentrations used) were not suitable and further setup is still needed.

DISCUSSION

Colorectal cancer (CRC) is the third most common cancer worldwide and the fourth most common cause of cancer-related death, and its incidence has risen steadily. Both environmental and genetic factors have been correlated with its pathogenesis and progression. In particular, extensive research has suggested that nutrition may play both a causal and protective role in the development of CRC (4).

Indeed, on one hand, findings from epidemiologic and experimental investigations have linked consumption of several foods and nutrients to the risk of colorectal neoplasia (4). On the other, different evidence suggest that a combination of drugs, nutritional supplements, diet and exercise could, instead, prevent the risk of disease (5). In particular, the use of polyphenols and phytochemicals – a class of biomolecules known as potent micronutrients – has been proposed as promising approach in the prevention of various diseases, such as diabetes, cardiovascular diseases, and cancer. (65)

How these molecules are effective on CRC prevention? And which is the molecular mechanism behind? Different lines of evidence suggest that these biomolecules can modulate, directly or indirectly, cell signaling and protein posttranslational modification (PTMs) (6). Among these, the reversible PTM induced by nitric oxide (NO), so called S-nitrosylation, has been linked to cancer and affected by phytochemicals. *S*-nitrosylation is the covalent addition of an NO moiety to the sulfhydryl group of a reactive cysteine (S-H), generating an S-nitrosothiol (S-NO) (66). As above mentioned, *S*-nitrosylation is a reversible reaction; mammalian cells have, indeed, developed (and evolutionarily conserved) enzymatic systems able to catalyze the opposite reaction, *denitrosylation*. The main mechanism responsible for protein denitrosylation is based on the chemical transfer of the NO group from S-nitrosylated proteins (PSNO) to low-molecular weight thiols, such as glutathione (GSH) and Coenzyme A (CoA) in a reaction called *trans*-nitrosylation, which generates S-nitrosoglutathione (GSNO) and S-nitroso-CoA (SNO-CoA). Intracellularly, GSNO and SNO-CoA levels are controlled by two enymes: S-nitrosoglutathione reductase (GSNOR) and SNO-CoA reductase (SCoR). Therefore, by denitrosylating their subtrates, GSNOR and SCoR, indirectly regulate PSNO levels. Notwithstanding many pieces of evidence argue for *S*-nitrosylation being involved in

cancer onset and progression, the role of denitrosylases in these processes still needs to be clearly defined.

In this scenario, the aim of this thesis was to evaluate the bioactive effects of *Opuntia Ficus Indica* (OFI) waste material (namely the cladodes) on the modulation of NO signaling, with particular focus on the effect on GSNOR expression system. *Opuntia Ficus Indica* is a species native of Mexico, which belongs to the *Cactaceae* family. The cladodes of wild and domesticated *Opuntia Ficus Indica* have been used for centuries for nutritional and medicine purposes and are traditionally considered healthy nutritional sources for preventing chronic diseases such as diabetes, cardiovascular diseases, metabolic syndrome, obesity, or aging, as well as infectious or neurodegenerative diseases. Since several years, scientific research has been interested in deepening the knowledge on this plant, in order to better understand their nutritional and therapeutic properties. Indeed, in literature it's well characterized that *Opuntia ficus indica's* cladodes gel extract is particular rich in bioactive compounds (contained both in the fruit and in other parts of the plant) with promising – but, so far, only suggested – biological properties in terms of beneficial effects on human health.

Moreover, there is also an interest in their development for economical and food security purposes, as they easily grow in arid desert area. Indeed, given the growing interest of the society and the European Commission (Directive 2008/98 / EC on waste: Waste Framework Directive) in using waste materials as a source of nourishment for industrial costs and food waste reduction, this study is also aimed at testing the possible use of waste cladodes from OFI for possible applications in cancer research. In particular, I used OFI's gel (extracted from the cladodes and characterized at the ENEA Research Laboratory of Rome) and tested its potential anticancer effect in the CaCo2 human adenocarcinoma cell line.

The ENEA lab characterization of OFI gel shown that it is enriched in macro-elements, such as potassium (28.06% ± 0.52), calcium (3.03% ± 0.06) and magnesium (2.04% ± 0.11). Moreover, results shown that OFI matrix have a significant polyphenol content and antioxidant activity (Table 5). However, antioxidant activity could be the results of two mechanisms: the pool of biomolecules in the gel matrix could have antioxidant

properties themselves; or alternately act as an oxidant molecule and induce the activation of antioxidant pathway that mediate the effect. In order to unveiling the mechanism behind, activation of the master regulator of antioxidant and redox response, the transcription factor NRF2 was analyzed. NRF2 is a transcription factor activated by oxidative stress upon detachment from its adaptor protein Keap1. Once activated, it binds the antioxidant-responsive element (ARE) in the promoter region of many antioxidant genes and induces their transcription. In cancer cells it has been observed a constitutive activation of Nrf2, which leads to the acquisition of the so-called "redox adaptation" and chemoresistance. (67) Results in figure 13. shown that OFI treatments, do not affect the activation of NRF2 pathway. qPCR analysis, indeed, shown no modulation of mRNA of NRF2-target genes, such as hMOX1, NQO1 or GCL. This result suggests intrinsic antioxidant properties of the pool of biomolecules, but further analyses are necessary to confirm it.

It is well documented that OFI pads extract induce apoptosis in colon cancer cells HT-29 and cytotoxicity in different tumor cell lines. (46) Based on this evidence, the viability of Caco2 cells were assessed after the treatment with both the gel and flour extract of OFI cladodes. However, results from our study suggest that 6 to 56 h treatments with OFI [flour gel (0 to 50 ug/mL) and fresh matrix (0 to 5000ug/mL)], do not affect cancer cell viability (Figure 14-15), suggesting no cytostatic or cytotoxic effects. The apparent discrepancy with what reported in the literature could be probably attributable to different extraction methods, and also to the part of the plants used.

As mentioned above, the correlation between polyphenols, *S*-nitrosylation and cancer progression is still under investigation and further studies are necessary to understand the mechanism underneath. So far, there is no study that has investigated the role of OFI on GSNOR expression or S-nitrosylation. Therefore, the aim of this thesis was to shed light on this aspect. To do so, GSNOR expression was analyzed after different OFI concentrations. Results from Western blot analysis indicate that GSNOR protein levels decrease after OFI treatment in a dose- and time-dependent manner (Figure 16-17) , suggesting an effect of Opuntia Ficus Indica on NO signaling. Moreover, our results indicate that GSNOR decrease does not occur at transcriptional level (Figure

18) , but rather depends on the activation of autophagy (Figure 19) as a response to OFI extracts by still unknown mechanisms.

GSNOR decrease, such as the one observed in this study, is a condition previously correlated with aging and cell senescence.(68) Moreover, data obtained from the ENEA's laboratory, suggest that OFI gels exert anti-inflammatory properties. These evidence, together with data obtained in our study, prompted us to postulate that the pool of (still not fully identified) molecules contained in OFI gel might synergistically exert anticancer effect, such as by promoting cellular mechanisms (such as senescence) which counteract proliferation.

In addition, to recapitulate a more physiological condition of OFI absorption, we took advantage from the property of CaCo2 cells to form adherent monolayers. These are, indeed, good model to simulate drugs absorption. Therefore, with the aim to mimic a more physiological epithelial barrier model of human gut, we cultured adherent monolayers of Caco2 cells for 21 days. Next, to induce epithelium barrier dysfunction in vitro, we applied lipopolysaccharide (LPS) (64) and evaluated any modulation of OFI gel extract on i) inflammatory response and ii) GSNOR levels. Unfortunately, we were not able to induce an inflammatory response, thus making us realize that our experimental conditions (i.e., LPS concentrations used) were not appropriate and further setup is still needed.

Notwithstanding this incident, our results lead us to propose in the future to: i) address the role of GSNOR in the effects induced by OFI extracts in experimental models of cancer and inflammation, and ii) identify the bioactive molecule(s) responsible for the beneficial effects induced OFI extracts.

These findings might pave the way for developing OFI-based functional foods (such as juices or supplements),or producing OFI-based nutraceuticals from waste materials: two sectors of the industrial chain which might have tremendous impact on the economy and food safety of many arid and semi-arid regions of the world.

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