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Harmful effect of oxysterols in the gut and at systemic level: modulatory activity of olive oil phenolics

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Contents

Abstract

A healthy and proper diet is essential to prevent the onset of various diseases, as with the diet we can also introduce harmful substances.

Among others, the oxysterols are particularly relevant; they can be absorbed from the diet and are the oxidized products of cholesterol present in cholesterol-containing foodstuffs after cooking, processing and storage, such as meat, fish, dairy products and eggs. They can be also generated endogenously from either the spontaneous or enzymatic oxidation of cholesterol.

Oxysterols have been shown to exert several in vitro and in vivo biochemical activities, physiological but mostly pathological. In particular, they may induce cytotoxicity, cell death (necrosis and apoptosis), pro-oxidant and pro-inflammatory effects in different cells and tissues and have been linked with the onset and development of major chronic diseases.

Only in the last few years, oxysterols have been shown to interfere with the homeostasis of the human digestive tract, demonstrating their involvement in the pathogenesis of human inflammatory bowel disease and colon rectal cancer.

Experimental studies suggest that the ingestion of compounds with antioxidant action, such as phenolics, is able to counteract the oxidative stress and inflammatory response in the intestine and inhibit the onset of the main related diseases.

Extra virgin olive oil polyphenols in particular, recognized for their strong antioxidant property and related anti-inflammatory action, may display this effect in the intestinal lumen where they concentrate before absorption, preventing intestinal diseases.

The first aim of this study was to evaluate the ability of different extra virgin olive oil phenolic extracts to counteract the pro-oxidant, pro-apoptotic and pro-

inflammatory action of oxysterols in the human colon adenocarcinoma cell line (Caco-2) and the molecular mechanism involved.

Oxysterols treatment, depending on time of exposure and concentrations used, significantly altered the cellular redox status (ROS production increase, GSH decrease, GPX activity enhancement), induced an oxidative damage to the membrane lipid fraction (MDA production increase) and cell death (Caspase 3 increase); cytokines production (IL-6, IL-8) was indicative of a pro-inflammatory effect. Phenolic extracts were able to significantly counteract the oxysterols harmful effects, at least in part by modulating intracellular signaling pathways (Akt/PKB, MAPK) involved in the cellular response to oxidative stress, apoptosis and inflammation.

Several studies in animals and humans have shown that dietary oxysterols, after digestion can be absorbed from the gut and transported into the circulation within chylomicrons and other lipoproteins. Furthermore, the presence of oxysterols in plasma can derive from the oxidation of endogenous cholesterol through enzymatic or spontaneous reactions.

Oxysterols have been found at increased levels in the plasma of hypercholesterolemic subjects and have been linked with the atherosclerotic process.

In this context the second part of this study was specifically focused on the protective effect of pure olive oil phenolics, which can be found in the blood stream after absorption (hydroxytyrosol, tyrosol, and homovanillc alcohol), against the pro-oxidant and pro-inflammatory activity of oxysterols in blood cells (PBMCs).

It was demonstrated that all phenolic compounds tested, HT, TYR and HVA were able to inhibit oxysterol-induced pro-inflammatory cytokines production.

According to the hypotesis that pro-inflammatory cytokine release may be induced by changes in intracellular redox status, it was observed that, in human

PBMCs treated with the oxysterols mixture, simple phenols were also able to inhibit ROS production as well as to suppress both redox-based MAPK phosphorylation (JNK, p38).

These data suggest that olive oil phenolic extracts may significantly contribute to preserve the integrity of intestinal mucosa against oxidative damage and inflammation related disorders and simple phenols found in the blood after metabolism and absorption counteract the oxysterols-induced inflammatory effect at systemic level.

1. Introduction

1.1 Oxysterols

Oxysterols are a group of 27-carbon-atom cholesterol oxidation products (COPs) that may be generated in the blood, cell and tissues through enzymatic or non-enzymatic reactions, but are also absorbed from the diet **(Addis 1986, Smith 1987, Leonarduzzi et al. 2002, Jessup et al. 2005, Poli et al. 2009)**.

COPs have been studied and identified since 1966 when they were first detected in human atheromatous plaques **(Brooks et al. 1966)**. In the 90s, isotope dilution mass spectrometry enabled the study of oxysterols in human plasma **(Breuer et al. 1990, Dzeletovic et al. 1995)**. Since then, 7α-hydroxycholesterol (7α-HC), 7β-hydroxycholesterol (7β-HC), 7-ketocholesterol (7-KC), 5α,6α-epoxycholesterol (5α,6α-EC), 5β,6β-epoxycholesterol (5β,6β-EC), 3β,5α,6β-cholestanetriol (α-triol), 24-hydroxycholesterol (24-HC), 25-hydroxycholesterol (25-HC) and 27-hydroxycholesterol (27-HC) and many others have been found in human biological samples.

Oxysterols are structurally identical to cholesterol, but with one or more additional oxygen containing functional groups (such as alcohol, carbonyl or epoxide groups) **(Brown et al. 2009)**.

Cholesterol (Figure 1) is composed of three regions: a hydrocarbon tail (also called the lateral chain), a ring structure region with four hydrocarbon rings (A, B, C, and D), and a hydroxyl group.

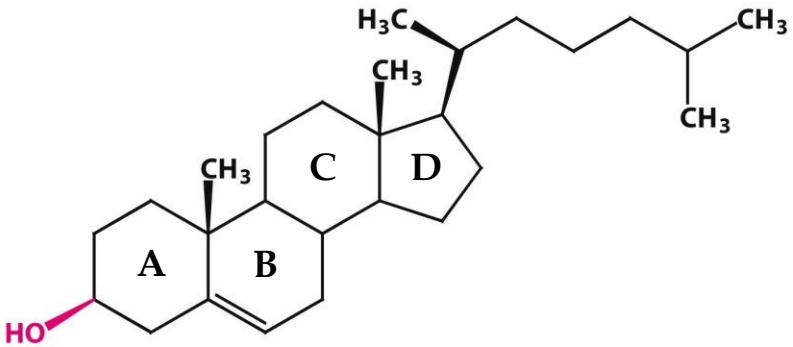


Figure 1: Molecular structure of cholesterol

Oxidation can occur on the ring structure and on the lateral chain. The double bond present on the B hydrocarbon ring can be the target of free radical attacks, and therefore positions 4, 5, 6, and 7 are the most sensitive sites of spontaneous oxidation **(Vejux et al. 2009)**.

In general, biological oxysterols fall into two main categories: those oxygenated on the sterol ring, mainly at the 7-position (i.e. 7-KC and 7α/β-HC) and those oxygenated on the side-chain (i.e. 24-HC, 25-HC and 27-HC). Generally, ring-oxygenated sterols tend to be formed non-enzymatically, whereas side-chain oxygenated sterols usually have an enzymatic origin. However, there are exceptions to this rule; for example 25-HC and 7α-HC can be produced by both enzymatic and non-enzymatic routes **(Gill et al. 2008)**.

Endogenous formation of oxysterols mainly occurs through autoxidation of cholesterol (Figure 2).

Cholesterol is attacked by reactive oxygen species (ROS) abstracting an allylic hydrogen atom at C-7 of the sterol ring. The radical generated can react with oxygen to form a cholesterol peroxyl radical (COO·), which further reacts abstracting hydrogen and generates the relatively stable cholesterol 7α/β-hydroperoxides (7α- and 7β-OOHC) **(Brown et al. 2009)**. At this point, hydroperoxides may continue oxidizing non-enzymatically, in fact, in the presence of trace levels of transition metals, they are further decomposed to 7α/β-alkoxy radicals (CO·), which in turn can undergo further reactions to generate 7α/β-hydroxycholesterols and 7-ketocholesterol. These are the major non-enzymatically generated oxysterols that are present in most tissues. Cholesterol hydroperoxides can be also reduced to epoxycholesterols enzymatically **(Brown et al. 1999, Brown et al. 2009)**.

Of note, some oxysterols of endogenous origin are exclusively produced via enzymatic reaction (Figure 2). 24-, 25- and 27-HCs are generated by enzymatic side-chain hydroxylation of cholesterol **(Romer et al. 2006)**. Sterol 27-hydroxylase (CYP27A1) and cholesterol 24-hydroxylase (CYP46A1) are P450 enzymes

expressed in liver and macrophages, and neural cells of the brain and retina, respectively **(Brown et al. 2009)**. They catalyse the hydroxylation reactions to form 27- and 24-HCs. Cholesterol 25-hydroxylase (Ch25 h) is the enzyme responsible for generating 25-HC and it is expressed at very low levels. Nevertheless, it is very interesting, since its product (25-HC) regulates the sterol regulatory element binding protein (SREBP) for cholesterol synthesis **(Russell 2000)**. Non-enzymatic oxidation of 25-HC has also been described **(Smith 1987)**, although the main source of this oxysterol is the enzymatic oxidation. Similarly, 7α-HC can be enzymatically generated by 7α-hydroxylase and 7-ketonedehydrogenase **(Smith 1996)**.

In addition, there are some enzymes (5α,6α-epoxydase) that reduce hydroperoxides to 5,6-ECs. These ECs can be further converted to their triol end products via hydration in an acidic environment **(Tai et al. 1999)**.

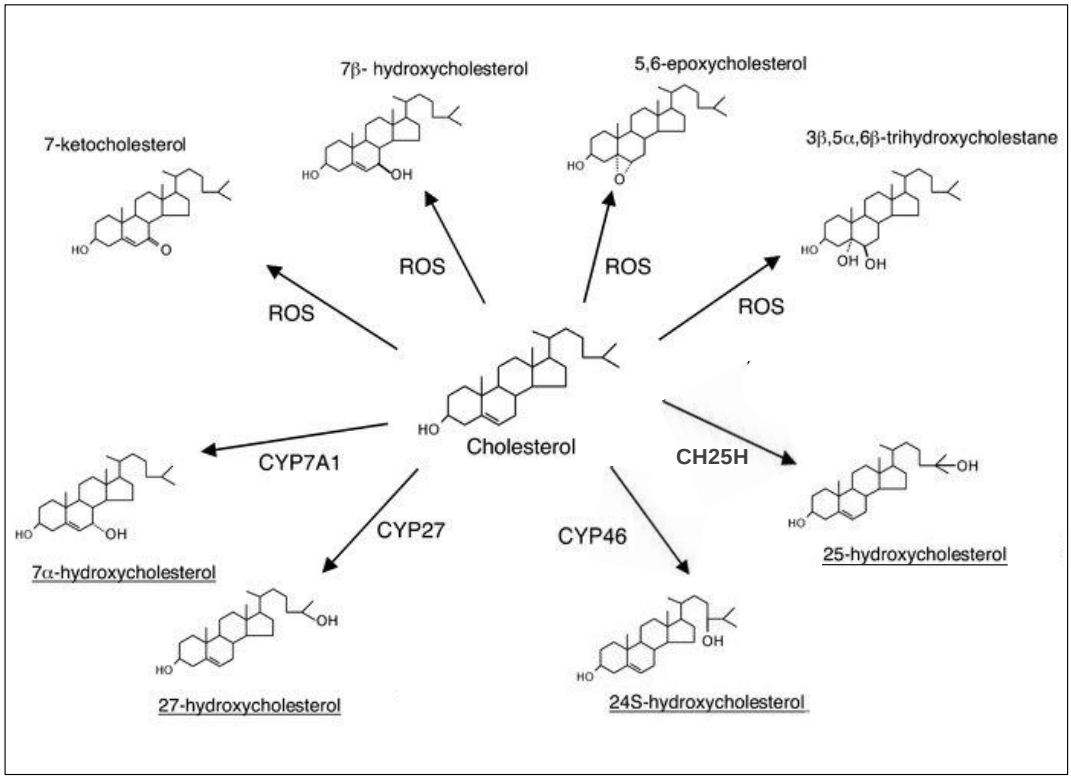


Figure 2: Enzymatic and non-enzymatic oxysterols production

Relatively high levels of oxysterols are present also in various foodstuffs, notably cholesterol-rich foods such as dairy products, milk, eggs, dried egg powder, clarified butter (or ghee), meat products, and dried or stored fish. Specifically, for example, the amount of COPs in meat products ranges from 0.1 μg/g (beef) to 18.7 μg/g (mortadella), while in seafood products up to 19.4 μg/g (Brazilian Sardines) and 33.6 μg/g (anchovies), in dried egg pasta ranges from 44 to 52 μg/g. For butter and spread, the content of oxysterols is around 13–27 μg COPs per gram and up to 259 μg/g for ghee **(Otaegui-Arrazola et al. 2010)**. Generally, products containing cholesterol are susceptible to oxidation, especially dehydrated foodstuffs subjected to radiation or submitted to high temperatures, as well as those that are cooked in the presence of oxygen **(Guardiola et al. 2002, Vejux et al. 2008)**. Indeed, under these conditions, alimentary cholesterol is exposed to numerous reactive oxygen species (ROS), such as singlet oxygen (O_2^-), which can contribute to the generation of cholesterol hydroperoxides, hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), and ozone (O_3). Consequently, when food is stored for lengthy periods in an environment other than in a vacuum, the generation of oxysterols markedly increases.

The most commonly detected oxysterols (Figure 3) in processed foods are 7-oxygenated sterols (7-KC, 7α-HC, 7β-HC), 5,6-oxygenated sterols (5α,6α-EC, 5β,6β-EC, cholestane-3β,5α,6β-triol), as well as 25-HC, 19-HC, 20α-HC, 3β-hydroxy-5α-cholestane-6-one, and 3β,5α-dihydroxycholestane-6-one, which are present in smaller amounts **(Guardiola et al. 2002, Vejux et al. 2008)**. Their concentrations have been quantified in the 10–100 μM range **(Kanner 2007)**. With regard to their percentage distribution, Plat and co-workers showed that, on heating cholesterol for 3 h at 180°C, about 95% oxysterols were produced, of which nearly 40% was 7-ketocholesterol, 35% the two epoxides, and 25% the two hydroxides **(Plat et al. 2005)**.

Lipid oxidation reactions not only usually cause food spoilage from the sensory point of view, but also induce chemical changes that might compromise their safety for consumption **(Otaegui-Arrazola et al. 2010)**.

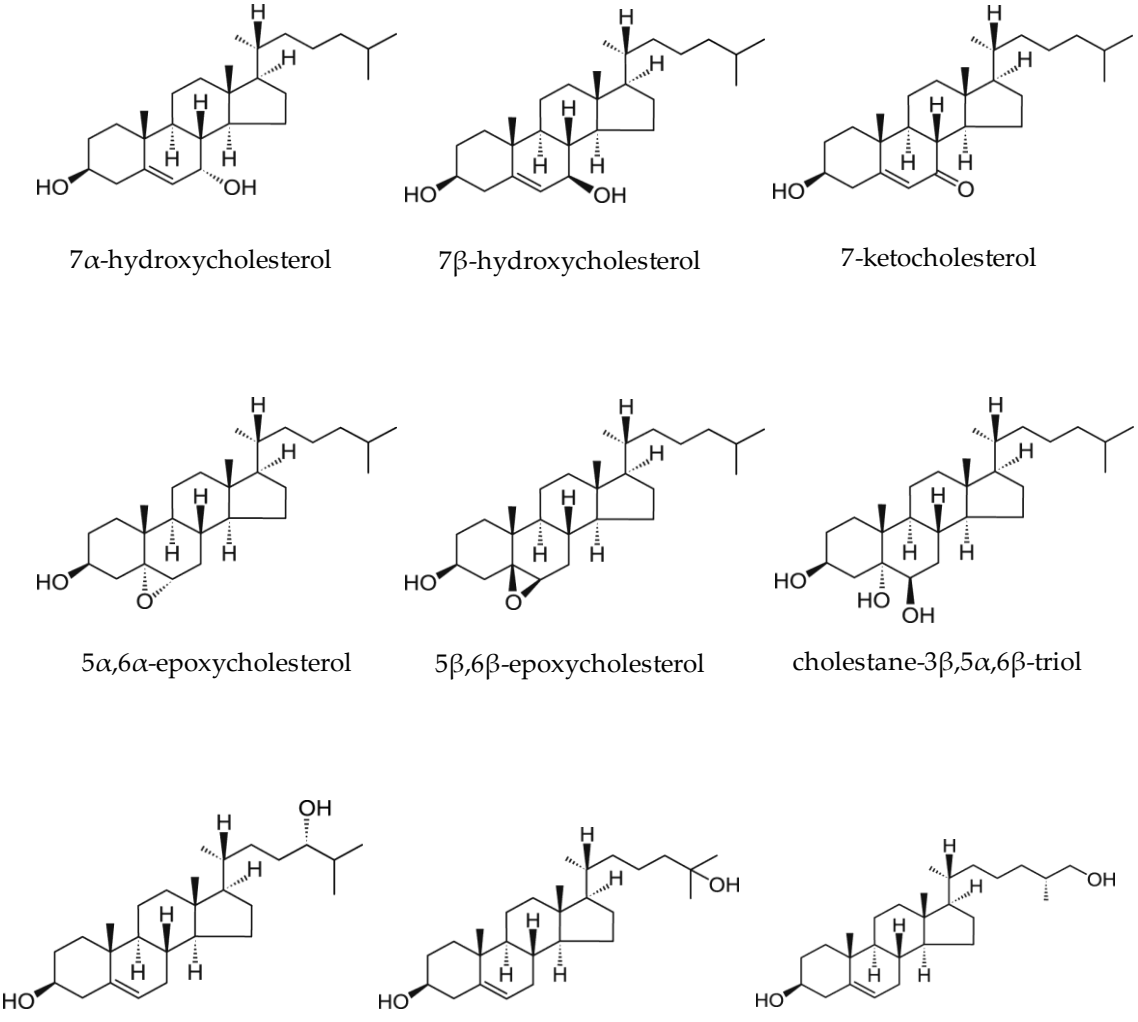


Figure 3: Main endogenous and dietary oxysterols

1.1.1 Oxysterols absorption

Several studies in rats, mice, hamsters and rabbits have shown that oxysterols incorporated through the diet or excreted by the liver where cholesterol is oxidized enzymatically and non-enzymatically can be absorbed in human intestine and distributed in tissues **(Otaegui-Arrazola et al. 2010)**.

The absorption of COPs is lower than that of cholesterol, probably due to the lower solubility of COPs in micelles, the lower susceptibility to esterification in enterocytes and the cytotoxic effects in mucosal cells, which could explain the reduced lymph flow in rats that had been given COPs **(Osada et al. 1994)**. Also, each type of COP is absorbed to different degrees; 7β-HC, 7-KC and 5α,6α-EC are detected in greater amounts than 5β,6β-EC and 25-HC, which are not quantified in some studies done on rabbits and healthy humans **(Osada et al. 1994, Linseisen et al. 1998, Vine et al. 1998)**. Interestingly, contrary to 7β-HC, the 7-KC is slightly absorbed and rapidly metabolized by the liver **(Schweizer et al. 2004)**.

A correlation exists between serum levels of COPs and their concentration in the diet. In rabbits that had been fed with COP-enriched diet, increased concentrations of COPs in plasma were found compared to those in the control diet group **(Vine et al. 1998, Ubhayasekera et al. 2010a)**. Also, a diet containing highly oxidized lipids has been found to significantly increase the contents of oxycholesterols in meat, liver and plasma of chickens **(Ubhayasekera et al. 2010b)**. These authors concluded that meat products from animals fed a diet containing higher levels of oxidized lipids may result in higher ingestion of oxycholesterols by humans.

Similarly, studies carried out in healthy humans have shown that after a diet containing 400 mg of α-EC, serum α-EC can be detected, in comparison with the control group in which α-EC was not quantified **(Staprans et al. 2003)**. Linseisen and Wolfram have found that the intake of Parmesan cheese and salami (providing 0.84 mg of unesterified COP and 2.64 mg of COP acyl esters) raises plasma COP levels. Free oxidized cholesterol concentration increased three hours

after the meal, but with very high interindividual variation. In contrast, total COP levels in plasma were up to 100-fold higher 6–8 h after the intake of salami and Parmesan, while the variation was lower **(Linseisen et al. 1998)**.

Hence, in several studies, the sterol oxidation concentrations found in plasma and tissues are higher than those that would be predicted from the concentrations in the test diets **(Linseisen et al. 1998)**.

Despite their lower absorption compared to cholesterol, oxysterols are absorbed more quickly in the intestine, have faster plasma clearance and are quickly collected by tissues. Oxysterols in human plasma or serum may vary from about 1 μM (0.05% of total cholesterol) in healthy subjects to 20-30 μM (0.5-0.75% of total cholesterol) in diseased individuals, but much higher concentrations of plasma oxysterols have also been reported **(Schroepfer 2000)**.

Part of the absorbed oxysterols is secreted back into the intestinal lumen by transporters ABCG5 and ABCG8 which are a subfamily of ATP-binding cassette transporters. Oxysterols might be pumped back into the gut through this route **(Hovenkamp et al. 2008)**. However, there have not been any specific studies about the implication of ABCG5 and ABCG8 in oxysterol transport.

1.1.2 Esterification and distribution

Oxysterols are mainly absorbed as esters in the intestinal tract, in fact, most of them measured in plasma and tissues are predominantly esterified **(Linseisen et al. 1998, Staprans et al. 2003)**, suggesting that they are good substrates for acyl-coA cholesterol acyl transferase (ACAT) in cells and lecitin cholesterol acyl transferase (LCAT) in the circulation **(Gill et al. 2008)**.

Once the ACAT in the enterocyte reacts with oxysterols, they are incorporated to chylomicrons and later to Very Low Density Lipoproteins (VLDL), Low Density Lipoproteins (LDL) and High Density Lipoproteins (HDL) **(Staprans et al. 2003, Staprans et al. 2005)**. Some studies have also shown that oxysterols can be transported by albumin and oxysterols in plasma have been found also in free form **(Guardiola et al. 2002).**

Hence, oxysterols can be transported to different cells of the organism.

COPs amounts in chylomicrons, LDL and HDL increased 2 h after a COP-enriched meal (400 mg of α-EC), while little α-EC was detected in VLDL. COPs remained in chylomicrons until 8 h, but at 72 h the circulating α-EC was found mainly in LDL, and less in HDL **(Staprans et al. 2003)**. This showed that the oxidized sterol products increased much more in LDLs than in other lipoproteins after the intake of a COP test diet. These results suggest that COPs may play an important role in atherogenesis.

7α/β-HCs and 7-KC are the most quantified oxysterols in lipoproteins and their concentrations are greater than those found in the test diets, suggesting that they are also synthesized endogenously **(Linseisen et al. 1998, Vine et al. 1998)**.

1.1.3 Further reactions or metabolism

Oxysterols can be metabolized or degraded to other compounds, mainly in the liver. Oxysterols are found to be further oxidized or reduced by enzymes. Some of them are key enzymes of the bile acid biosynthesis, such as 7α-hydroxylase and 27-hydroxylase **(Ryan et al. 2009)**. CYP27A1 can metabolize 7-KC to 27-hydroxylated-7-ketocholesterol and further to water-soluble metabolites which can be eliminated from cells **(Lyons et al. 2001, Larsson et al. 2007)**. On the other hand, 7-KC can also follow a reduction route, being converted to 7β-HC by 11β-hydroxysteroid dehydrogenase type1 **(Schweizer et al. 2004, Larsson et al. 2007)**. Cholesterol sulfotransferase enzyme (SULT2B1b) usually sulfates the 3β-hydroxyl group of cholesterol, but oxysterols also have been found to be substrates of this enzyme **(Fuda et al. 2007)**. SULT2B1b is expressed in retina, skin, platelets, liver and other tissues; hence, this route could be an important oxysterol excretion pathway **(Vogelsang et al. 1998, Higashi et al. 2004, Fuda et al. 2007)**.

1.1.4 Excretion

Most of the oxysterols can be only eliminated from cells through specific membrane lipid transporters as a consequence of their hydrophobicity. Apart from the ABCG5 and ABCG8 transporters that pump back oxysterols into the intestine, there are other ATP-binding cassette transporters involved in oxysterol excretion. ABCA1 and ABCG1 are mainly located in the macrophages and liver to transport oxidized sterols and other molecules out of the cell **(Brown et al. 2009)**.
Some of the absorbed oxysterols are taken up from the circulation into tissues, but the relative abundance of individual oxysterols is different in diet, serum and tissues, indicating dietary oxysterols are modified in vivo by differential uptake, metabolism and elimination. The extent to which dietary sources contribute to tissue oxysterol levels is expected to depend heavily on the specific types as well as the levels of their intake **(Brown et al. 2009)**.

1.1.5 Biological activities

Oxysterols have been shown to exert several *in vitro* and *in vivo* biochemical activities of both physiologic and pathologic relevance **(Brown et al. 1999, Schroepfer 2000)**. Compared to cholesterol, the presence of a further oxygen group makes these compounds more polar and more easily diffusible through cell membranes **(van Amerongen et al. 1989, Kan et al. 1992)**. As a consequence, they are much more reactive than the parent compound, promoting and sustaining cytotoxicity, mutagenicity, carcinogenicity, atherogenicity, inflammation, fibrosis, and programmed cell death in a number of cells and tissues **(Smith et al. 1989, Lordan et al. 2009, Vejux et al. 2009)**.

It is well established that oxysterols are involved in some physiological processes, they exert important functions as regulators of the expression of genes involved in lipid and sterol biosynthesis **(Brown et al. 1974, Kandutsch et al. 1974)**, as substrates for the formation of bile acids **(Danielsson et al. 1984)** and as mediators of reverse cholesterol transport whereby excess cholesterol is returned to the liver for excretion **(Bjorkhem et al. 1994, Lutjohann et al. 1996, Norlin et al. 2000, Bjorkhem 2007)**.

However the oxysterols are potentially involved in the onset and progression of major chronic diseases which inflammation, but also oxidative damage and to a certain extent cell death, are hallmarks and primary mechanisms of progression. These include atherosclerosis, diabetes, kidney failure, ethanol intoxication **(Guardiola et al. 1996, Guardiola et al. 2002, Sottero et al. 2009)**, neurodegenerative diseases such as Alzheimer's **(Bjorkhem et al. 2006, Cao et al. 2007, Vaya et al. 2007)** and Parkinson's diseases **(Bosco et al. 2006, Rantham Prabhakara et al. 2008)** and recently they have been associated with human inflammatory bowel diseases (IBD) such as ulcerative colitis and Crohn's disease **(Biasi et al. 2009, Mascia et al. 2010, Biasi et al. 2013a)**.

Therefore it is now suggested that oxysterols might contribute to the development of numerous other degenerative diseases such as multiple sclerosis **(Diestel et al. 2003, Leoni et al. 2005)**, age-related macular degeneration **(Malvitte et al. 2006)**, osteoporosis **(Liu et al. 2005)** and cataract **(Girao et al. 1998)**.

In vitro and in vivo studies have also demonstrated an association between different types of oxysterols and the development and progression of cancer of the colon, lung, breast and bile ducts **(Jusakul et al. 2011)**.

1.2 Oxysterols in the gut

The gastrointestinal tract is constantly exposed to dietary oxidized food compounds produced during the reactions that occur during processing and storage of foods **(Kanner 2007)**. Unintended ingestion of COPs is increasing in recent years due to frequent use of industrialized foods, longer shelf-life, and the consumption of questionable quality fast food **(Vicente et al. 2012)**.

The colon mucosa provides a physiological barrier against potentially pathogenic components of the diet, as well as against intestinal flora present in the lumen. Colon epithelial cells counteract potential insults through a highly complex network of cell signals that control the intestinal immune response, inflammatory status and mucosal wound healing **(Biasi et al. 2008)**.

Persistent inflammation, due to bacterial pathogens or genetic predisposition, induces massive recruitment of inflammatory cells, which lead to derangement of mucosal barrier. Cytokines, chemokines and antimicrobial mediators, such as prostaglandins and eicosainoids derived from lipid metabolism, are actively involved in this process. Dietary lipids have an important patho-physiological impact on mucosa function: they influence membrane fluidity, which may alter cell signaling from the receptor system, immune response, inflammatory reactions and cell death **(O'Sullivan et al. 2003, Mills et al. 2005)**. A large body of evidence from epidemiological studies indicates that dietary lipids, such as polyunsaturated fatty acids (PUFAs) or cholesterol oxidation products, and intestinal microflora, are the main responsible for the production of oxidized species in the colon **(O'Sullivan et al. 2003, Whiting et al. 2005)**. Excessive oxidative cell reactions together with reduced antioxidant tissue defences are prominent features in the pathogenesis of several chronic gastrointestinal diseases **(Bruce et al. 2000, Keshavarzian et al. 2003)**.

Oxysterols have very recently been shown to potentially interfere with homeostasis of the human digestive tract, by promoting and sustaining

irreversible damage of the colonic epithelial layer **(Mascia et al. 2010),** these products could contribute to the pathogenesis of the intestinal epithelial barrier dysfunction that occurs in human inflammatory bowel diseases (IBD), the gut pre-neoplastic disorders, like ulcerative colitis and Crohn's disease **(Biasi et al. 2009)**, and colon cancer **(Biasi et al. 2008, Jusakul et al. 2011)**.

Studies from O'Sullivan and colleagues have shown that 25-HC, 5α, 6α-EC, 5β, 6β-EC or 7β-OH added to undifferentiated Caco-2 cells determined a reduction in cell viability and 25-HC exhibited the greatest cytotoxicity **(O'Sullivan et al. 2003)**.

The same research group further challenged undifferentiated Caco-2 cells for 24 h in the presence of 30 μM 5β, 6β-EC, 7β-HC, or 7-KC, showing that cell viability, in terms of neutral red uptake, had decreased by 36, 11, and 17%, respectively **(Ryan et al. 2005)**.

In another study 30 μM 7β-HC inhibited cell growth by 50% in undifferentiated Caco-2 cells. However 7β-HC, didn't alter the cell cycle, didn't activate caspase-3 and 8, but there was a significant increase in the activity of caspase-9 and DNA fragmentation. Treatment with a caspase inhibitor (Z-VAD.fmk) didn't delay apoptosis induced by 7β-HC. These results suggest that the 7β-HC induces cell death in Caco-2 through a mechanism not mediated by caspases **(Roussi et al. 2005).**

The same research group further challenged undifferentiated Caco-2 cells 12 and 24 h in the presence of 30 μM 7β-HC, showing a decrease of cell viability and mitochondrial membrane potential, as well as the release of cytochrome c. However, 7β-HC didn't affect the expression of the two members of Bcl 2 family proteins Bcl-2 and Bax, which are known to regulate mitochondrial membrane permeabilization. They showed that this oxysterol was also able to induce lysosomal membrane permeabilization, and suggested that cytotoxic effect of 7β-HC could be explained by a direct action on membrane integrity of both mitochondria and lysosomes **(Roussi et al. 2007)**.

Biasi and colleagues showed that a mixture of oxysterols stemming from the heating of dietary cholesterol, comprising 7-KC, 7α-HC, 7β-HC, 5α, 6α-EC and 5β, 6β-EC, led differentiated human epithelial colonic cells (Caco-2 cells) to apoptotic death. Specifically, they observed, in cells treated with oxysterol mixture, a net decrease in mitochondrial potential followed by cytochrome C release into the cytosol and caspase-3 activation was found to be 30–40% higher than in untreated cells **(Biasi et al. 2009)**. The observed pro-apoptotic effect was prevented when up-regulation of Nox1 was inhibited; Nox1 is the intestinal isoform of NADPH oxidase, and its activation increases intracellular ROS steady-state, in fact the key event in the sterol-induced up-regulation of programmed death in differentiated Caco-2 cells was an enhanced production of reactive oxygen species consequent to the marked activation of colonic NADPH oxidase. Indeed, both mitochondrial Caco-2 cells were prevented by the pharmacologic [diphenylene iodonium (DPI)] inhibition of NOX activity **(Biasi et al. 2009)**.

Very recently, the likely role of phagocytic NOX (Nox2) as well as that of isoforms highly expressed in the gastrointestinal mucosa (Nox1, Duox2) in the pathogenesis of IBD and colon carcinogenesis have been comprehensively analyzed and described **(Rokutan et al. 2008)**.

The cytotoxicity of 7-KC at different concentrations (0-120 μM) and incubation times (4-24 h), in intestinal epithelial cells (Caco-2 cells) was also evaluated. 7-KC showed a deleterious effect upon the mitochondrial compartment after 24 h of exposure (120 μM), as well as upon mitochondrial membrane potential when incubated at all concentrations (12/24 h), but without affecting the endo/lysosomal compartment.

Cells incubated with 7-KC (30–60 μM) showed an increasing trend for total RNA in the G1 phase population. Accordingly, these changes were associated to a decreasing trend of total RNA content in the G2 population. On the other hand, the opposite trend was observed when cell cultures were exposed to 120 μM of 7-KC, where total RNA content in the G1 population was significantly lower than

the values found even in control cultures. This reflecting the negative effects of this compound upon the main functional cell population, impairing protein synthesis **(Alemany et al. 2012)**.

Biasi and colleagues determined the effects in colon cancer Caco-2 cells (undifferentiated) of the same representative dietary mixture of oxysterols previous cited, that was used in concentrations corresponding to low (30 μM), moderate (60 μM) and high (90 μM) daily dietary intake. Only the 90 μM concentration appeared to be necrogenic, and to exclude any necrosis and to achieve the maximum biological effects, all subsequent experiments used 60 μM concentration of oxysterols, added individually or as a mixture. 60 μM oxysterol mixture exerted a slight apoptotic effect in terms of caspase-3/-7 activities, compared to control and regarding the single compounds, only 5α,6α-EC and 7α-HC values were significantly higher than controls, and caspase-3/7 activation in 7β-HC-treated cells was significantly increased compared to both controls and oxysterol mixture.

Caco-2 cells incubated, with the oxysterol mixture showed also, a slight but significant NOX1 increase compared to controls. As regards cell treatment with individual components, similarly to findings for caspases' activity, 5α, 6α-EC and 7β-HC were significantly more efficient than the oxysterol mixture in inducing NOX1 activity. In particular, NOX1 activation by 7β-HC was significantly elevated compared to controls, and also compared to the oxysterol mixture. Consequent on the stimulation exerted on NOX1 activity, a burst in ROS production by Caco-2 cells was observed, the trend being similar to that detected for NOX1. The oxysterols-dependent apoptosis in Caco-2 cancer cells was mediated most likely through a strong early generation of ROS upon activation of the colonic NADPH oxidase complex NOX1. Indirect confirmation of these results comes from the significant protection against pro-apoptotic events afforded by cell pre-treatment with specific NADPH oxidase inhibitor (DPI).

In this study the production of IL-8 and MCP-1, the two well known pro-inflammatory cytokines with chemoattractant property for neutrophils and macrophages, respectively, have been investigated.

The only slight increase in IL-8 was observed in the oxysterol mixture treatment group, but neither 7β-HC, nor 5α, 6α-EC, nor 7α-HC enhanced cytokine synthesis. Interestingly, pre-treatment of cells with DPI only caused 0-20% inhibition of IL-8 production. Unlike IL-8, cell treatment with 7β-HC significantly induced MCP-1, which was also increased by cholesterol and oxysterol mixture. However, DPI pre-treatment wasn't able to reduce MCP-1 induction to control values in all experimental groups.

Notably, the partial or even absent inhibition of MCP-1 by DPI suggests a secondary role of NADPH oxidase in the induction of inflammation by oxysterols in this colon cancer cytotype. Other mechanisms are probably involved in the induction of inflammatory chemokines in undifferentiated Caco-2 cells **(Biasi et al. 2013a)**.

The same dietary oxysterol mixture (30 μM), but also one of its component, i.e. 7β-HC (4.4 μM), also exhibited a strong pro-inflammatory effect on differentiated Caco-2 cells, significantly up-regulated the expression of IL-8 mRNA levels, that was followed by a net increase in synthesis of the cytokine. This is one of the most important cytokines involved in intestinal inflammation, exercises chemo-attraction and promotes neutrophil migration into the epithelial layer. The sterols' pro-inflammatory effect seems to be mediated by enhanced activation of NOX1.

Data reported in the paper, in fact, show that the representative dietary mixture of oxysterols exerts a colitic effect through up-regulation of the colonic NADPH oxidase isoform NOX1. Furthermore induction of IL-8 in Caco-2 cells by oxysterol mixture was prevented by pretreating the cells with the NOX inhibitor DPI.

The interactions among the various components of the oxysterol mixture in inducing IL-8 expression and synthesis were also investigated. Each oxysterol was added singly to the differentiated Caco-2 cells at the same concentration as when

applied in the mixture. Components 7-KC, 5α, 6α-EC, 5β, 6β-EC, and 7α-HC didn't produce the IL-8 values observed in the cells treated with the oxysterol mixture when used singly. In contrast, 7β-HC, used singly, enhanced IL-8 mRNA and IL-8 protein, respectively 2.1 and 1.3 times more than did the oxysterol mixture.

The probable interaction of 7β-HC with the other components of the oxysterol mixture was then examined: the cells were treated with 7β-HC plus 7-KC, with 7β-HC plus 5α, 6α-EC, with 7β-HC plus 5β, 6β-EC, or with 7β-HC plus 7α-HC, and IL-8 expression and synthesis were evaluated. Three of the four oxysterols appeared to slightly modulate the IL-8 up-regulation exerted by 7β-HC. Only combined treatment with 5β, 6β-EC and 7β-HC induced IL-8 mRNA and protein levels similar to those reached in cells treated with the oxysterol mixture.

Cell treatment with the oxysterol mixture significantly up-regulated the expression of other pro-inflammatory interleukins in addition to IL-8, namely IL-1α, IL-6, MCP-1, and IL-23; in addition, the oxy-mixture increased the mRNA cellular levels of the multifunctional peptide TGF-β1 and of two membrane receptors involved in modulating innate intestinal immunity, namely TLR2 and TLR9. Cell treatment with 7β-HC increased the expression of all the molecules induced by the oxy-mixture, except for TLRs 2 and 9; its effect appeared more marked than that of the oxysterol mixture as regards the frankly pro-inflammatory cytokines IL-1α, IL-6, MCP-1, and IL-23 **(Mascia et al. 2010).**

Bai and colleagues investigated the effect of oxysterols, including 25-HC and 7β-HC, on IL-1β-induced IL-8 production in Caco-2 cells. Pre-treatment of Caco-2 cells with 25-HC significantly enhanced IL-1β-induced IL-8 expression at both mRNA and protein levels. However, 7β-HC showed very little effect on IL-8 production. IL-β is an important mediator in intestinal inflammation, promotes IL-8 production, which can be modulated by a number of factors, including oxidative stress.

Furthermore, pre-treatment with 25-HC, followed by IL-1β stimulation, enhanced IL-8 promoter activity beyond that observed with IL-1β alone. These results suggest that 25-HC enhances IL-1β-induced IL-8 production, possibly by enhancing promoter activity **(Bai et al. 2005)**.

Cholesterol oxidation products were shown able to induce expression and synthesis of transforming growth factor beta1 (TGFβ1), an event which can be detrimental or beneficial, essentially depending on its actual intensity. TGFβ1 is fundamental to maintain the intestinal epithelial cell homeostasis through its control action on cell proliferation, differentiation and apoptosis, but its deregulation has been observed in several chronic human diseases, including ulcerative colitis, Crohn's disease and colon carcinoma **(Biasi et al. 2007)**.

Certain lipid oxidation products may enhance the activity of several inflammatory cytokines, including TGFβ1, and thus these products may exert pro-inflammatory and pro-fibrogenic effects within the colon mucosa **(Leonarduzzi et al. 1997, Poli 2000)**.

The inflamed intestinal mucosa is characterized by uncontrolled release of cytokines and of oxidative species, which chiefly arise from respiratory burst of activated phagocytes. The dysfunction of TGFβ1 signaling is widely involved in the overproduction of inflammatory mediators in IBD **(Marek et al. 2002, Kader et al. 2005, Monteleone et al. 2005)**. In the advanced stages of IBD, fibrosis is the major complication; it manifests as strictures in Crohn's disease and as colonic shortening in ulcerative colitis. Fibrosis is mediated by intestinal fibroblasts, located at the interface between the epithelium and the lamina propria, which achieve the myofibroblast phenotype in the presence of TGFβ1**(Biasi et al. 2007)**.

Neoplastic cells tend to become less susceptible to the growth regulatory effects of TGF-β1 mainly because of reduced expression and/or activity of TGF-β1-specific receptors, as reported for many human cancers including colon cancer. Consequently, a sustained increase of TGF-β1 in the intestinal mucosa, like that caused by inflammatory processes and/or high dietary intake of animal fat, might

become crucial for the progression of a neoplastic clone. In fact, this proapoptotic and pro-differentiating cytokine could eliminate neoplastic cells still susceptible to TGF-β1's antiproliferative action (TGF-β1 receptor-positive cells), indirectly favoring the expansion of TGF-β1 resistant ones (TGF-β1 receptors deficient or negative cells). The actual concentration of TGF-β1 in the colonic mucosa undergoing neoplastic transformation is still debated, and the phase of the relevant carcinogenetic process in which a reduced susceptibility to this antiproliferative molecule first occurs has not been precisely established yet. However, no doubt that TGF-β1 level and activity may be up-regulated in cells of the macrophage lineage by animal fat oxidation products, such as oxysterols and aldehydes. But phagocytes as well as fibroblasts constitutively express TGF-β1 and are accumulating in tumor-associated stroma. Thus, up-regulation of this cytokine system within colonic tumor-associated stroma by excess dietary intake of cholesterol and n-6 polyunsaturated fatty acids appears as a primary mechanism of cancer progression at least in neoplastic lesions of the digestive tract **(Biasi et al. 2008)**.

In vitro studies demonstrated the effect of oxysterols on cytokine release by intestinal cells cultured alone. However, physiologically, the response of the intestinal epithelium to external agents occurs in the presence of dendritic cells (DCs) migrating within the subepithelial space **(Iwasaki et al. 2001, Rimoldi et al. 2005)**. Furthermore, when the epithelial barrier is broken, cholesterol and other compounds may affect the activity of cells such as DCs in tissues deeper within the intestinal wall.

Chalubinski and colleagues showed the effects of oxidised cholesterol on epithelial barrier functions in human intestinal epithelium co-cultured with dendritic cells. In particular, they showed that 7-ketocholesterol decreased the transepithelial electrical resistance (TEER) of Caco-2 cell co-cultured with DCs. The loss of TEER, reflecting monolayer integrity, was consistent with the decrease of tight junction protein ZO-1 mRNA expression **(Chalubinski et al. 2014)**.

Oxidized cholesterols, as previously shown, are able to aggravate local inflammatory processes by inducing the release of pro-inflammatory cytokines, such as IL-8, IL-1β and TNF-α **(Lemaire et al. 1998, Dushkin et al. 2006, Mascia et al. 2010)**. However, 7-KC, a strong proinflammatory agent, has been demonstrated to have no effect on IL-10 synthesis in Caco-2 cells cultured alone **(Alemany et al. 2013)**. Otherwise, Chalubinski and colleagues demonstrated that 7-ketocholesterol diminished IL-10 mRNA expression in Caco-2 cell induced by the presence of DCs **(Chalubinski et al. 2014)**.

A previous study of the same research group has demonstrated, in a Real-time Cell Electric Impedance Sensing system, the ability of 7-KC and 25-HC to decrease the integrity of intestinal epithelial monolayer **(Chalubinski et al. 2013)**.

1.3 Oxysterols in the circulatory system

Hypercholesterolemia has long been related to the pathogenesis of atherosclerosis, but the actual mechanism by which cholesterol favours both the initiation and the progression of this disease is still debated. The epidemiologic evidence supporting cholesterol as a primary risk factor of cardiovascular diseases has not been validated thus far by available biochemical data, which clearly indicate cholesterol as a poorly reactive molecule. Conversely, especially in the last 10–15 years, a number of experimental studies have consistently shown that cholesterol oxidation products, in particular oxysterols, possess a much higher biochemical reactivity than that of the parent compound **(Sottero et al. 2009)**.

This evidence prompted several research groups to focus on the potential modulation of atherosclerotic disease by the oxysterols, that have been detected in human plasma of hypercholesterolemic individuals, especially in LDL subfractions, in human macrophages/foam cells and atherosclerotic plaque **(Chang et al. 1997, Brown et al. 1999)**.

Oxysterols in plaque are derived both non-enzymically, either from the diet and/or from in vivo oxidation, or (i.e. 27-HC) are formed enzymically during cholesterol catabolism **(Brown et al. 1999)**.

The formation of atherosclerotic lesions is a complex process in part mediated by inflammatory and oxidative mechanisms **(Steinberg et al. 1989, Ross 1999, Steinberg 2002)**. Oxidative changes occurring to low density lipoproteins (LDL) are widely regarded as playing a critical role in fibrotic plaque initiation and development. Several studies have shown that following endothelial insult by various stimuli, significant amounts of LDL micelles are retained in the sub-intimal spaces of the arterial wall where they undergo oxidative modifications leading to the formation of oxidized LDL (oxLDL) **(Salvayre et al. 2002)**. Several lipid-derived bioactive molecules are generated in LDL during oxidation, for example, oxidized phospholipids, lysophospholipids, aldehydes, and mainly

cholesterol oxidation products that accumulate in the vascular subintimal space **(Sevanian et al. 1995, Chang et al. 1997)**. Oxidized LDL, in fact, are not recognized by LDL receptors, they are instead taken up by scavenger receptors on macrophages of arterial walls **(Stocker et al. 2004)**.

The accumulation of these compounds within the vascular subintimal space is unanimously recognised as being involved in the recruitment of circulating monocytes, their transformation into macrophages, the subsequent uptake of modified lipoprotein particles and the eventual formation of foam cells. These lipid-filled cell undergo apoptosis leading to an abnormal accumulation of cell debris in the lesion, which contributes to the instability of plaque and the atherothrombosis **(Sottero et al. 2009)**.

Specifically, COPs are involved in various key steps of the atherogenic process: (1) endothelial cell dysfunction (increasing permeability); (2) adhesion and transmigration of monocytes (expression of cell adhesion molecules, chemokines and cytokines); (3) generation of foam cells (differentiation of monocytes to macrophages); (4) macrophages and smooth muscle cell interaction and extracellular matrix over-production; (5) inflammation and fibrotic cap formation; (6) vascular apoptosis and extracellular matrix degradation **(Poli et al. 2009)**.

Some studies have indicated that oxLDL, known to contain high levels of oxysterols, and some oxysterols identified in atherosclerotic lesions or entering retinal pigmentary epithelial cells through choriocapillaris **(Brown et al. 1999, Colles et al. 2001, Malvitte et al. 2006)** are relevant in the induction of oxidative processes resulting from an overproduction of ROS. ROS are either free radicals such as superoxide anion ($O_2^{\cdot -}$) and hydroxyl radical (·OH), reactive anions containing oxygen atoms such as H_2O_2 and peroxynitrite ($ONOO^-$), and/or secondary radicals [alkyl (R·), alkoxyl (RO·) and peroxyl (ROO·)], which are chemically activated by ROS following the oxidizing activity of ROS on unsaturated fatty acids, carbohydrates, and/or proteins; they can, in turn, contribute to producing free radicals. It is worth noting that these ROS have

important side effects on vascular wall cells at various stages of the development of atherosclerosis as well as on retinal and neuronal cells. Indeed, they favour certain major pro-atherosclerotic events: proliferation of smooth muscle cells; enhanced accumulation of oxLDL in macrophages and smooth muscle cells as well as subsequent formation of foam cells; loss of endothelial cell reactivity; peroxidation of membrane lipids and accumulation of lipid oxidation products such as malondialdehyde and oxysterols resulting from the autoxidation of cellular cholesterol **(Guardiola et al. 2002)**; induction of cell death **(Colles et al. 2001)**.

In atherosclerotic apolipoprotein E-deficient mice, it has also been reported that some oxysterols (7-KC, 7β-HC, and 5β,6β-EC) induced the activation of macrophage NADPH oxidase, arachidonic acid release, and superoxide anion production, which contribute to enhancing cell-mediated oxidation of LDL, and that vitamin E was able to counteract these different events **(Rosenblat et al. 2002)**.

In vitro, under treatment with 7-KC, 7β-HC, 5β,6β-EC, 5α,6α-EC, and 25-HC, important oxidative processes, sometimes associated with a complex mode of cell death with some of the characteristics of apoptosis, have also been described on various cell types **(Miguet-Alfonsi et al. 2002, Pedruzzi et al. 2004, Lemaire-Ewing et al. 2005)**. Thus, on 7-KC treated U937 cells, an abrupt drop in reduced glutathione associated with an increased production of ROS preceding caspase activation has been reported **(Lizard et al. 1998)**. In human aortic smooth muscle cells, 7-KC also induces oxidative stress associated with apoptotic events, and it has been demonstrated that this specific effect of 7-KC is mediated by a robust upregulation (3-fold from the basal level) of Nox-4, an ROS-generating NAD(P)H oxidase homologue **(Pedruzzi et al. 2004)**.

Oxysterol mixtures, in atheroma-relevant proportions, also display pro-oxidative activities. Thus, when 7β-HC is combined with 5β,6β-EC the decrease in the glutathione level in U937 cells is significantly greater than with 7β-HC alone **(O'Sullivan A et al. 2005)**. Subsequent experiments showed that when 7-KC is

administered to cells together with another oxysterol, 7β-HC, the generation of ROS in J774-A1 murine macrophages is markedly attenuated **(Biasi et al. 2004)**. It has been proposed that competition among oxysterols, apparently at the level of NADPH oxidase, diminishes the ROS induction and direct toxicity that is evoked by specific oxysterols. Moreover, in the J774-A1 murine macrophage cell line, with a biologically representative oxysterol mixture [5-cholestene-3β,7α-diol (10.2%), 5-cholestene-3β,7β-diol (6.7%), cholestane-5α,6α-epoxy-3βol (10.3%), cholestane-5β,6β-epoxy-3β-ol (24.3%), cholestane-3β,5α,6β-triol (10.0%), 5-cholestene-3β-ol-7-one (31.0%), and 5-cholestene-3β,25diol (7.3%)], which approximates the proportions of oxysterols found in LDL from human plasma, the mRNA level of manganese superoxide dismutase was markedly increased after 24-h exposure. This over expression of manganese superoxide dismutase mRNA may serve as an important adaptive response of oxysterols to regulate oxygen species involved in the apoptotic death of macrophage/foam cells. However, in certain conditions, anti-oxidative effects of oxysterols have also been described. Thus, oxidized cholesterol in oxLDL may be responsible for the inhibition of lipopolysaccharide (LPS)-induced nitric oxide production in J774-A1 murine macrophages **(Liu et al. 1998)**.

Oxysterols have been shown to up-regulate the expression of various inflammatory molecules, including adhesion molecules, growth factors, cytokines and chemokines. In human monocytic cells, 7-KC, 7β-HC and 25-HC (at concentration 50 μM) are potent in vitro inducers of a variety of molecules, such as IL-β, TNFα, MIB-1β and MCP-1 **(Prunet et al. 2006)**.

25-HC was found to up-regulate expression and synthesis of IL-1β in human macrophages **(Rosklint et al. 2002)**, and IL-1β is also markedly induced by 7-KC, 7α-HC, and, in particular by 7β-HC in human promonocytic cells U937 and U4 **(Lizard et al. 1997, Lemaire-Ewing et al. 2005).**

Of the oxidative agents present in oxLDLs that may be responsible for inflammatory processes, various oxysterols are of primary interest **(Lemaire-**

Ewing et al. 2005). When human monocytes, monocyte-derived macrophages (THP-1 cells), and porcine retinal pigment epithelial cells were exposed to a series of different oxysterols (7-KC, 7β-HC, 24-HC, 25-HC, or cholestan-3β,5α,6β-triol), all of them had a tendency to stimulate IL-8 production, but 25-HC was the most potent one **(Liu et al. 1997, Joffre et al. 2007).** It should be noted that on human mononuclear cells, the pro-inflammatory activity of 25-HC was enhanced in hypoxic conditions **(Rydberg et al. 2003)**. Moreover, on human promonocytic leukemia cells (U937) and on the J774-A1 murine macrophage cell line, a biologically representative oxysterol mixture 7α-HC (5%), 7β-HC (10%), 5α,6α-EC (20%), 5β,6β-EC (20%), cholestan-3β,5α,6β-triol (9%), 7-KC (35%), and 25-HC (1%) induced both expression and synthesis of key chemokines for monocytes/macrophages, particularly MCP-1 **(Leonarduzzi et al. 2005)**, and up-regulated TGF-β1 expression, thus playing a pivotal role in smooth muscle cell chemoattraction and differentiation into myofibroblast-like structures **(Leonarduzzi et al. 2001)**.

In addition, on human umbilical venous endothelial cells (HUVECs), 7α-HC, 7β-HC, 7-KC, and 25-HC also enhanced the expression of adhesion molecules involved in the recruitment of immunocompetent cells, such as ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), and E-selectin **(Lemaire et al. 1998, Naito et al. 2004)**.

Increased levels of IL-6 have been found in macrophagic cells treated with 25-HC or 27-HC **(Yudkin et al. 2000, Rosklint et al. 2002)**, IL-6 transcript was also increased by 7-KC treatment in human aorta SMCs **(Sung et al. 2009)**, and synthesis of TNFα, a strong pro-inflammatory cytokine, was stimulated by 22-hydroxycholesterol in human peripheral monocytes and in monocytic THP-1 cells **(Landis et al. 2002)**. 25-hydroxycholesterol induced the production of TNFα and IL-1 in adherent human peripheral blood mononuclear leukocytes **(Feng et al. 2001)**.

In relation to atherosclerosis, 7-KC significantly stimulated vascular endothelial growth factor (VEGF) production as well as NO synthesis in rat and human vascular SMCs. Indeed these oxysterols-mediated effects might contribute to the growth of atherosclerotic plaque **(Dulak et al. 2001)**.

Oxysterols may also enhance the inflammatory response by inhibiting secretion of IL-10, a key anti-inflammatory cytokine. Pre-incubation of macrophages with atorvastatin, an anti-inflammatory agent, increased the IL-10 level by 41%, while 7-KC and 25-HC inhibited secretion of IL-10 respectively by 48 and by 55% **(Dushkin et al. 2006).**

Another interesting effect that these compounds show in sustaining inflammation is the promotion of monocyte differentiation into macrophages. It has recently been shown that a mixture of oxysterols detectable in human plaques (total amount 20 μM), up-regulates expression and synthesis of the scavenger receptor CD36 with a consequent uptake of oxLDL **(Leonarduzzi et al. 2008)**.

1.4 Mediterranean diet and health

Eating is one of life's great pleasures, and there are many time-tested diets that are compatible with good health. The Mediterranean diet (MD) is considered one of the healthiest dietary models for its contribution to a favourable health status and a better quality of life **(Willett et al. 1995, Serra-Majem et al. 2006)**. The traditional MD has entered the medical literature following publications by the legendary Ancel Keys and his colleagues of results from their "Seven Countries Study", initiated in the late 1950s **(Keys 1980)**.

Several complementary dietary patterns have existed around the Mediterranean basin for centuries, in fact, there is no single MD; indeed more than 20 countries have a coastline on the Mediterranean Sea, and more countries are included in what is termed the Mediterranean region. There are many variations to the MD, due to social, political and economic differences between these countries **(Perez-Lopez et al. 2009)**.

Mediterranean dietary patterns (MDPs) are characterized by the consumption of cereals (preferably as whole grains), legumes, nuts, vegetables and fruits, in high amount and frequency; MDPs also include reduced consumption of fish or seafood, white meat and eggs, moderate to small amounts of poultry and dairy products and low ethanol intake, usually in the form of wine. The principal source of dietary lipids is olive oil, the key component of a MD **(Estruch et al. 2013)**. In addition, MDPs also include the practicing of physical activity in order to maintain a healthy physical and mental status **(Bach-Faig et al. 2011)**. On the contrary, the "Western" diet, diffused in industrialized countries, presents a higher intake of animal-derived foods (saturated fats), eggs, sweets, desserts and a lower eating of fruits, vegetables (fibers and micronutrients) and whole cereals **(Lairon 2007)**. Furthermore, in the MD, the consumption of traditional and local food products is a strength, respecting the seasonal availability and the biodiversity of food **(Del Chierico et al. 2014)**.

Since the first data from the seven countries study, several studies in different populations have established a beneficial role for the main components of the MD **(Serra-Majem et al. 2006)**.

Vegetables, fruits and nuts are the most important source of fibers and chemical compounds, like flavonoids, phytosterols, vitamins, terpenes and phenols, which give protection against oxidative processes, hence reducing the incidence of cardiovascular diseases (CVD) **(Garritano et al. 2005, Ninfali et al. 2005, Panico et al. 2005, Ortega et al. 2006)**. Furthermore, the consumption of olive oil, as the predominant fat intake, provides high oleic acid content and polyphenols, which have antiatherogenic, antioxidant and anti-inflammatory effects, reducing the cholesterol/high density lipoprotein (HDL) ratio and the concentration of the oxidized low density lipoprotein (LDL) **(Covas et al. 2006, Jakobsen et al. 2009, Lopez-Miranda et al. 2010)**. Furthermore, olive oil has high levels of monounsaturated fatty acids (MUFAs) and a higher MUFA/saturated FAs (SFAs) ratio, which contribute to the protective effects **(Serra-Majem et al. 2009, Bos et al. 2010)**. Polyunsaturated FAs (PUFAs), contained in fish (i.e., eicosapentaenoic and docosahexaenoic acids), regulate hemostatic factors and provide protection against cardiac arrhythmias, cancer and hypertension and play a role in the preservation of cognitive functions **(Ortega et al. 2006, Grosso et al. 2014, Grosso et al. 2014)**. Typically, MDPs include low-glycemic index and low-glycemic load foods, derived from the whole grain and other fiber-rich product intake, which have been associated with a lower risk of diabetes, mainly type 2, coronary health diseases (CHD) and cancer, while refined grain has been linked to the risk of diabetes, obesity, CHD and other chronic diseases **(Barclay et al. 2008, Haas et al. 2009)**. The water-rich dairy products characteristic of the MD, such as yoghurt and cheese, are well tolerated by lactose-intolerant subjects. In addition, lactic acid bacteria, contained in the yoghurt, improve gastrointestinal (GI) health and immune response, displaying probiotic benefits **(Ortega et al. 2006)**. Moreover, the consumption of yoghurt may induce positive modifications in the gut microbiota,

which have been associated with a reduction of colon cancer risk indices **(Bartram et al. 1994)**.

Another important aspect in the MD, frequently ignored, is the low sodium intake; in fact, high sodium intake has been linked with high blood pressure, while consumption of salt-preserved foods has been connected with higher risk of stomach cancer, CHD and mortality **(Bibbins-Domingo et al. 2010)**. Some of the beneficial effects of the MD in human diseases have been attributed to the polyphenols contained in red wine. Indeed, the antioxidant activity of these compounds may also be responsible for cytoprotective and cardioprotective action **(Chiva-Blanch et al. 2013)**.

The MD is associated with reduced risk of morbidity (particularly atherosclerosis, cardiovascular disease, and certain types of cancer) and mortality and this has partially been explained by the consumption of extra virgin olive oil **(Cicerale et al. 2010)**.

1.5 Olive oil

The Olive (Olea europaea L.) is a small, even green tree, which belongs to the family Oleaceae and is native to tropical and warm temperate regions of the world, but it is well adapted for survival in extreme temperatures and periods of drought. The tree, famous for its fruit, also called the olive, is commercially important in the Mediterranean region as a prime source of olive oil **(Boskou et al. 1996)**. The tree is typically distributed in the coastal areas of the eastern Mediterranean Basin, the adjoining coastal areas of south-eastern Europe, western Asia and northern Africa as well as northern Iran at the south end of the Caspian Sea. Although olive is now cultivated in several parts of the world, the Mediterranean region still serves as the major production area accounting for about 98% of the world's olive cultivation **(Ryan et al. 1998)**.

According to estimates, the cultivation of olive tree dates back more than 7000 years. The olive tree has a long history of medicinal and nutritional values. The products of Olea europaea have been used as aphrodisiacs, emollients, laxatives, nutritives, sedatives, and tonics. Specific conditions traditionally treated include colic, alopecia, paralysis, rheumatic pain, sciatica, and hypertension **(Gilani et al. 2005)**.

The olive fruit is an oval-shaped drupe and possesses a typical size of 2-3 cm (width and length) and pulp per stone ratios of 3.0-6.5. The olive fruit is essentially made up of 3 parts, epicarp or skin, mesocarp or pulp and endocarp or stone. The epicarp (skin) is covered with wax; during the growth phase the skin colour turns from light green to purple and brown or black. The mesocarp, with a soft, pulpy flesh, accounts for 84-90% (of the total fruit mass) while the hard endocarp (stone) containing the seed or kernel may differ from 13 to 30% of fruit weight. The seed contains 2-4 g oil /100 g. Olive fruit weight may range from 2-12 g, although some varieties may weigh as much as 20 g **(Boskou et al. 2006, Niaounakis et al. 2006)**.

The growth and ripening of olive fruit is a long process, which takes about 5 months in usual climatic conditions. However, in cold climatic conditions, growth is slower. Olive fruit's average composition includes water (50%), protein (1.6%), oil (22%), carbohydrate (19.1%), cellulose (5.8%), inorganic substances (1.5%) and phenolic compounds (1-3%). Other important compounds present in olive fruit are pectin, organic acids, and pigments. The distribution and structure of the chemical constituents of olive fruit is complex and dependent on parameters including variety, cultivation practices, geographical origin, and the level of maturation and these parameters influenced also the quality of extra virgin olive oil **(Boskou et al. 1996).**

Extra virgin olive oil (EVOO) is obtained solely through physical means by mechanical or direct pressing of the olives under mild thermal conditions that do not lead to alterations in the oil composition. The olives are first crushed to form a pomace, then homogenized and pressed. This oil is not subjected to any treatment except washing, decantation, centrifugation and filtration. The oil produced from this first press is known as extra virgin olive oil and is of the highest quality. It contains also the highest levels of beneficial constituents **(Rafehi et al. 2012)**.

It is widely used for food preparations (as salad oil, cooking oil, in frying and pasta sauces), in cosmetics and the pharmaceutical industry. In the olive fruits, oil is mainly concentrated in the pericarp (96–98%). The formation and accumulation of oil in the drupe, a rich reservoir of many classes of lipids, is possibly the reason why the oil has a unique flavour and fragrance. The olive flesh components are transformed to the oil, which mainly consists of two components, namely saponifiables and unsaponifiables. The former, comprising triacylglycerols (TAG), partial glycerides, esters of fatty acids or free fatty acids and phosphatides, represent nearly 98% of the oil chemical composition, while the later, consisting of mainly minor components such as hydrocarbons (squalene), phytosterols (β-sitosterol, stigmasterol, and campersterol), tocopherols (α-tocopherol), carotenoids (β-carotene), coloring pigments (chlorophylls), aliphalic and triterpenic alcohols,

volatile compounds and phenolics (tyrosol and hydroxytyrosol), contribute around 1–2% of the oil composition **(Viola et al. 2009, Rafehi et al. 2012)**.

The oil triglycerides are mainly represented by monounsaturates (oleic acid), along with small amount of saturates and considerable quantity of polyunsaturates (mainly of linoleic acid) **(Aparicio et al. 2000)**.

Historically, the healthful properties of virgin olive oil were attributed to a high proportion of monounsaturated fatty acids (MUFAs) **(Tripoli et al. 2005)**. However, several seed oils (including sunflower, soybean, and rapeseed) rich in MUFA have been demonstrated to be ineffective in beneficially altering chronic disease risk factors **(Aguilera et al. 2004, Harper et al. 2006)**. In addition to MUFA, virgin olive oil contains a minor, yet significant phenolic component that other seed oils lack. Thus, the phenolic fraction of virgin olive oil has generated much interest regarding its health promoting properties. Subsequent studies (human, animal, *in vivo* and *in vitro*) have demonstrated that olive oil phenolics have positive effects on certain physiological parameters, possibly reducing the risk of chronic disease development, such as CVD, neurodegenerative disease and cancer **(Martin-Moreno et al. 1994, Nicolaiew et al. 1998, Stark et al. 2002, Visioli et al. 2002, Carluccio et al. 2003, Boskou et al. 2006, Covas et al. 2006, Meza-Miranda et al. 2015, Rodriguez-Morato et al. 2015)**.

Olive oil exerts potentially beneficial biological effects resulting from antioxidant and anti-inflammatory activities of its phenolic fraction **(Cicerale et al. 2012)**.

The consumption of olive oil can provide heart health benefits such as favourable effects on cholesterol regulation and LDL-cholesterol oxidation **(EFSAJournal 2011)**, as well as exerting anti-inflammatory, antithrombotic and antihypertensive vasodilatory effects **(Esposito et al. 2004, Covas 2007, Perez-Jimenez et al. 2007)**. However, there are many other benefits, including on haemostasis: platelet function, thrombogenesis and fibrinolysis **(Covas 2007, Lopez-Miranda et al. 2007)**.

Recent studies consistently support the concept that the Mediterranean diet and consumption of olive oil have been connected with a reduced risk of morbidity and mortality and compatible with healthier aging and increased longevity **(Martin-Pelaez et al. 2013)**. In countries where the population adheres to the MD, such as Spain, Greece and Italy, and olive oil is the principal source of fat, rates of cancer incidence (mainly breast, colorectal and prostate cancers) are lower than in northern European countries **(Lopez-Miranda et al. 2010)**.

1.6 Phenolic compounds in olive oil

The chemical composition of the phenolic fraction of olive oil has been studied extensively. Phenolic compounds of olive oil are conventionally called polyphenols, although not all of them are polyhydroxyl derivates **(Martin-Pelaez et al. 2013)**.

Phenolics can be grouped according to their similar chemical structure in the following groups:

- *Phenolic acids*. They can be divided into two subgroups; benzoic acid derivatives and cinnamic acid derivatives, such as gallic acid, protocatechuic acid, p-hydroxybenzoic acid, vanillic acid, caffeic acid, syringic acid, p- and o-coumaric acid, ferulic acid, and cinnamic acid **(Mannino et al. 1993, Servili et al. 2004)**.
- *Phenolic alcohols*. These compounds possess a hydroxyl group attached to an aromatic hydrocarbon group, hydroxytyrosol (3,4-dihydroxyphenyl-ethanol or 3,4-DHPEA,) and tyrosol (p-hydroxyphenyl-ethanol or p-HPEA) **(Montedoro et al. 1992)**. Their concentration is usually low in fresh oils but increases during oil storage **(Montedoro et al. 1992)** due to the hydrolysis of extra virgin olive oil secoiridoids.
- *Secoiridoid.* The most abundant are the dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol (3,4-DHPEA) or tyrosol (p-HPEA), (3,4-DHPEA-EDA or p-HPEA-EDA), oleuropein, an isomer of the oleuropein aglycon (hydroxytyrosol linked to elenolic acid) (3,4-DHPEA-EA), and ligstroside aglycon (tyrosol linked to elenolic acid) (p-HPEA-EA). p-HPEA-derivates and dialdehydic forms oleuropein and ligstroside aglycon were also detected as minor hydrophilic phenols of extra virgin olive oil **(Owen et al. 2000, Rovellini et al. 2002)**.
- *Hydroxy-isocromans*. This is a class of phenolic compounds recently characterized of extra-virgin olive oil and the presence of 1-phenyl-6,7-

dihydroxy-isochroman and 1-(39-methoxy-49-hydroxy) phenyl-6, 7-dihydroxy- isochroman has been shown in several samples **(Bianco et al. 2001)**.

- *Flavonoids*. These phenolic compounds contain two benzene rings joined by a linear three carbon chain. They can be further divided into seven subgroups. Luteolin and apigenin are the most important **(Rovellini et al. 2002)**.
- *Lignans*. The exact structure of this type of phenolic is not well understood but is based on the condensation of aromatic aldehydes. (+)-1-acetoxypinoresinol and (+)-1-pinoresinol were characterized as the most concentrated lignans in extra virgin olive oil **(Owen et al. 2000)**. These compounds are present in the pulp and in the woody portion of the seed **(Servili et al. 2004)**.

The phenolic acids were the first group of phenolic compounds found in extra virgin olive oil; these compounds together with phenyl-alcohols, hydroxy-isochromans and flavonoids **(Bianco et al. 2001)**, are present in small amounts in extra virgin olive oil **(Montedoro 1972)**, while secoiridoids and lignans are the most prevalent phenolic compounds.

The phenolic fraction of virgin olive oil is heterogeneous, with at least 36 structurally distinct phenolic compounds identified; not all phenolics are present in every virgin olive oil and there is variation in the phenolic concentration between oils (100–700 mg/kg) **(Cicerale et al. 2009)**. Such variation in the phenolic fraction among virgin olive oils is partially a result of an array of production factors including: variety of the olive fruit (cultivar), region in which the olive fruit is grown, agricultural techniques used to cultivate the olive fruit, maturity of the olive fruit at harvest, and olive oil extraction, processing, storage methods and time since harvest. Cooking methods have also been shown to alter phenolic concentrations in virgin olive oil. Finally, research has shown that the analytical method used to quantify the concentration of phenolic compounds present in

virgin olive oil has an influence on the reported concentration **(Cicerale et al. 2010)**.

Many different studies have deepened the topic regarding the factors that influence olive oil phenolics content, in particular the type of cultivar.

Olive polyphenols content depends both quantitatively and qualitatively from the olive variety. Different varieties, cultivated in the same environment and processed at a fixed ripening stage, produce EVOO with different total polyphenols content **(Vitaglione et al. 2013)**.

Gomez-Alonzo and colleagues **(Gomez-Alonso et al. 2002)** demonstrated that the concentrations of several phenolic compounds varied significantly among different Spanish virgin olive oil varieties. For instance, the total phenolic content of the four virgin olive oil varieties examined in the study were as follows: Arbequina 25.5 ± 1.85 mg/kg, Hojiblanca 33.6 ± 3.14 mg/kg, Picual 37.9± 4.64 mg/kg, and Cornicabra 39.2 ± 1.80 mg/kg.

Virgin olive oils from different cultivars have also been found to possess characteristic phenolic profiles. For example, the olive oil variety Santulhana was found to contain a high amount of luteolin, Lentisca was found to contain a high concentration of hydroxytyrosol and oleuropein, and Madural Fina was found to contain a high hydroxytyrosol/oleuropein ratio **(Vinha et al. 2005)**.

Another study investigated the phenol content of monovarietal olive oils obtained from different Sardinian cultivars. The total phenolic content was highly variable between cultivars: Bosana 440 mg/Kg, Semidana 340 mg/Kg, Nera 200 mg/Kg, Nocellara 323 mg/kg, Carolea 360 mg/Kg and Pizz'è Carroga 108 mg/Kg. The Bosana cultivar among others contains the highest amount of total polyphenols, and also of single compounds like, hydroxytyrosol, tyrosol and their derivatives **(Cerretani et al. 2006)**.

The phenolic constituents contribute to the organoleptic profile of raw olives and, consequently, to that of extra virgin olive oil **(Uylaser et al. 2014)**. They confer a

bitter, pungent taste and a strong, fruity flavour to the oil, indicating a high organoleptic quality **(Visioli et al. 2002)**.

Extra virgin olive oils with high phenol levels exhibit also a high stability, in fact its antioxidant capacity, contributes significantly to determine the shelf-life of the oil, preventing its autoxidation **(Fito et al. 2007)**.

Studies conducted thus far (including human, animal, in vivo and in vitro experiments) have demonstrated that olive oil phenolic compounds have positive effects on various physiological biomarkers, implicating phenolic compounds as partially responsible for health benefits associated with the Mediterranean diet. Furthermore, olive oil phenolic compounds have been shown to be highly bioavailable, reinforcing their potential health promoting properties **(Cicerale et al. 2010)**.

1.7 Metabolism and bioavailability of phenolics

The phenolic concentration and composition of EVOO are important parameters to be considered, but the degree to which these components are bioavailable (absorbed, metabolized, distributed and eliminated) is fundamental in understanding and evaluating the health benefits associated with such compounds. To achieve an effect in specific tissues or organs, olive oil phenolic compounds must be readily bioavailable **(Cicerale et al. 2012)**.

The metabolic fate of phenolic compounds after ingestion has been the subject of study by the scientific community to find out the mechanisms through which they exert their activity into the organism. It is known that polyphenols suffer strong metabolism phase I and phase II, in which they are hydrolysed (phase I) and later conjugated (phase II) into their glucuronidated, methylated and sulphated forms in order to be absorbed **(Manach et al. 2004)**. In the case of EVOO, studies have focused on some specific compounds such as hydroxytyrosol (HT), tyrosol (TYR) and oleuropein (OL), and they have been reported to be readly bioavailable and the most biologically active **(Cicerale et al. 2010)**.

After ingestion, olive oil polyphenols can be partially modified in the acidic environment of the stomach. Aglycone secoiridoids are subjected to a time-dependent hydrolysis in the acidic gastric environment, leading to an approximate 5-fold increase in the amount of free HT and 3-fold increase in free TYR, after only 30 min **(Corona et al. 2006)**. This decomposition of secoiridoid aglycones increases with increased gastric residency, although under normal pH conditions (pH 2.0) and normal physiological time frames (up to 4 h) some remain intact and enter the small intestine un-hydrolyzed **(Corona et al. 2006)**. In contrast, if the ingested secoiridoid is glucosylated it appears not to be subjected to gastric hydrolysis **(Vissers et al. 2002)**, meaning that phenolics such as the glucosides of oleuropein enter the small intestine unmodified, along with high amounts of free HT and TYR and remaining secoiridoid aglycones. A human study conducted in normal and

ileostomy subjects fed with olive oil polyphenols (OL and ligstroside aglycon, HT and TYR) has indicated that the major site for the absorption of olive oil polyphenols is the small intestine and the amount of phenolics absorbed was 55-66% **(Vissers et al. 2002)**. Tuck and colleagues demonstrated increased bioavailability of HT and TYR when administered as an olive oil solution compared to an aqueous solution. The differences in bioavailability have been suggested to be due to the high antioxidant content of virgin olive oil compared to water and this high antioxidant content may have protected the breakdown of phenolics in the gastrointestinal tract prior to absorption **(Tuck et al. 2001)**.

Following ingestion of EVOO, the levels of HT and TYR increase rapidly achieving a peak concentration at approximately 1 h in plasma **(Miro-Casas et al. 2003a)** and around 2 h in urine **(Miro-Casas et al. 2001a, Miro Casas et al. 2001b, Miro-Casas et al. 2003a)**, which supports the evidence that the small intestine is the major site of absorption for these compounds.

A low quantity of phenolics present in urine after ingestion would indicate that these phenolics are readily absorbed. Excreted phenolics (mainly in the form of HT and TYR) were determined to be 5–16% of the total ingested **(Vissers et al. 2002)**. Excretion of approximately 24% of administered TYR was demonstrated in a study by Miro-Cases and colleagues **(Miro Casas et al. 2001b)**. Finally, Visioli and colleagues **(Visioli et al. 2000b)** reported the excretion of administered HT and TYR to be between 30–60% and 20–22% of the total ingested by human subjects, respectively. The above findings demonstrate that humans absorb a significant portion (~40–95%, using HT and TYR as proxy) of the dietary olive oil phenolic compounds they consume **(Vissers et al. 2002)**.

The mechanism by which absorption occurs with regards to olive oil phenolic compounds remains unclear. However, the different polarities of the various phenolics has been postulated to play a role in the absorption of these compounds **(Vissers et al. 2002)**. For instance, the phenolics TYR and HT are polar compounds and their absorption has been postulated to occur via passive diffusion **(Manna et**

al. 2000). The polar but larger phenolic, oleuropein-glycoside may be absorbed via a different mechanism to TYR and HT. It has been proposed that oleuropein-glycoside may diffuse through the lipid bilayer of the epithelial cell membrane and be absorbed via a glucose transporter. Two additional mechanisms for oleuropein-glycoside absorption are potentially via the paracellular route or transcellular passive diffusion **(Edgecombe et al. 2000)**. The phenolics, oleuropein and ligstroside-aglycones are less polar and currently there are no data available on their mechanism of absorption. Further research is required to substantiate the mechanisms of absorption for these phenolics and further investigate the mechanisms for other phenolic compounds.

The analysis of human plasma and urine **(Miro Casas et al. 2001b, Miro-Casas et al. 2003a)** has also demonstrated that both HT and TYR are dose-dependently absorbed and are metabolized primarily to O-glucuronidated conjugates **(Visioli et al. 2000b, Caruso et al. 2001, Visioli et al. 2001, Miro-Casas et al. 2003b)**. HT also undergoes O-methylation by the action of catechol-O-methyl-transferase (COMT), and both homovanillic acid and homovanillyl alcohol (HVA) have been detected in human and animal plasma and urine after the oral administration of either EVOO or pure HT and TYR **(D'Angelo et al. 2001, Tuck et al. 2001, Tuck et al. 2002, Visioli et al. 2003, Miro-Casas et al. 2003b)**. A study conducted by Corona et al. about absorption, metabolism and microflora-dependent transformation of HT, TYR and their conjugated forms, such as Ol also shown similar results, in fact, both HT and TYR transferred across human Caco-2 cell monolayers and rat segments of jejunum and ileum, were subject to classic phase I/II biotransformation. The major metabolites identified were an O-methylated derivative of HT, glucuronides of HT and TYR and a novel glutathionylated conjugate of HT (HT-GSH). In contrast, there was no absorption of Ol in either model. However their studies have also demonstrated that secoiridoids, which appear not to be absorbed in the small intestine, undergo bacterial catabolism in

the large intestine with oleuropein undergoing rapid degradation by the colonic microflora producing HT as the major end product **(Corona et al. 2006)**.

Most recently, Garcia-Villalba et al. reported the presence of metabolites for the majority of olive oil phenolic compounds (i.e. secoiridoids, flavanoids and phenolic alcohols) in human urine, further suggesting that these compounds are metabolised and absorbed post-ingestion. For instance, the largest number of metabolites was produced from HT, oleuropein aglycone and oleocanthal, indicating significant postabsorption metabolism of these compounds. Conversely, the lowest number of metabolites came from TYR, luteolin, apigenin, pinoresinol and acetoxypinoresinol, suggesting that these compounds may have been excreted in faeces, destroyed in the gastrointestinal tract, excreted through another metabolic pathway or poorly absorbed **(Garcia-Villalba et al. 2010)**. In the case of poorly absorbed phenolic compounds, it has been suggested that these components may exert local antioxidant activities in the gastrointestinal tract and this proposal is supported by research demonstrating the free radical scavenging capacity of olive oil phenolics in both the faecal matrix and intestinal epithelial cells **(de la Torre 2008)**. Further, it is postulated that unabsorbed olive oil phenolics may exert antimicrobial activities in the gastrointestinal tract as has been demonstrated with tea and certain fruit phenolics **(Selma et al. 2009)**. However, further research is required to substantiate these and to gain a more thorough understanding of the metabolism of the various phenolic compounds present in EVOO.

1.8 Biological activities of olive oil phenolics

Many reviews, focused on the nutritional properties of EVOO phenolics, have been published in the last years **(Covas et al. 2006, Covas 2007, Fito et al. 2007, Corona et al. 2009, Covas et al. 2009, Cicerale et al. 2010, Cicerale et al. 2012)**.

Human and animal research (both *in vitro* and *in vivo*), conducted using some pure compounds typical of EVOO (HT, TYR, oleocanthal, etc) or EVOO polyphenol extracts, concurrently attributed to these compounds many health benefits, demonstrating their important biological activities and preventive effects in regards to the development of chronic degenerative diseases.

One of the main properties of olive oil phenolics contributing directly or indirectly to their beneficial health effects is their antioxidant capacity.

An excess of free radicals like reactive oxygen species (ROS), which are continuously formed by our organism as a result of normal metabolic process, and also enter our body through food or the environment, can cause oxidative damage to cellular biomolecules (i.e. lipids, DNA and proteins), increasing the risk of developing numerous chronic diseases such as atherosclerosis, cancer, CVD, IBD, chronic inflammation, stroke and other degenerative diseases **(Uttara et al. 2009)**.

Phenolic compounds in EVOO have been shown to exert beneficial effects on lipid oxidation, DNA oxidative damage and in general oxidative stress, *in vitro* and *in vivo*, and these activities have been shown to have a subsequent positive effect on disease risk **(Cicerale et al. 2009)**.

In experimental studies (*in vivo* and *in vitro*), phenolics have been shown to beneficially alter blood lipid composition **(Vissers et al. 2001, Visioli et al. 2005)**, inducing a significant increase in circulating HDL **(Mangas-Cruz et al. 2001, Marrugat et al. 2004, Weinbrenner et al. 2004, Covas et al. 2006)**, and in particular significantly decreasing LDL, total cholesterol and triglycerides **(Gimeno et al. 2002, Gorinstein et al. 2002)**.

In addition, different studies have demonstrated that an increased ingestion of olive oil phenolics was able to reduce and inhibit the oxidation of LDL, considered to be a major risk factor for the development of atherosclerosis and CVD **(Witztum 1994)**.

Polyphenols can also exert a protective effect against the oxidative damage to DNA, considered a precursor for human carcinogenesis **(Cooke et al. 2003)**. Research concerning DNA damage for example (as measured by the comet assay and 8-oxo-deoxyguanosine) showed that the intake of phenol-rich EVOO decreased oxidative DNA damage in vivo in humans by up to 30% **(Salvini et al. 2006, Machowetz et al. 2007)**. Animal and in vitro studies have also mirrored these human study findings **(Fabiani et al. 2008, Jacomelli et al. 2010)**.

In vitro studies have shown that olive oil has beneficial effects on cellular function **(Fabiani et al. 2006)**, for example modulating factors that are underlying for tumor formation and progression, such as deregulated cell proliferation and suppressed cell death **(Evan et al. 2001)**.

Epidemiology has shown an inverse correlation between an olive oil rich in polyphenols consumption and cancer in different sites, breast **(Martin-Moreno et al. 1994, Trichopoulou et al. 1995, Garcia-Segovia et al. 2006)**, pancreas **(Soler et al. 1998)**, oral cavity **(Franceschi et al. 1999)**, oesophagus **(Bosetti et al. 2003)**, colon-rectum **(Stoneham et al. 2000)**, prostate **(Hodge et al. 2004)** and lung **(Fortes et al. 2003)**.

A number of human studies have also demonstrated the beneficial effects of olive oil phenolics on other markers of oxidative stress **(Cicerale et al. 2010)**, such as F2-isoprostanes, lipid peroxides (LPO), and glutathione including oxidized glutathione (GSSG), reduced glutathione (GSH) and glutathione peroxidase (GPX). F2-isoprostanes are formed from the free radical oxidation of the cell membrane derived polyunsaturated fatty acid (FA), arachidonic acid. LPO is likely to be derived from oxidation of FA **(Ruano et al. 2005)**. Furthermore, the main biological role of glutathione is protecting an organism from oxidative

damage. In particular, depletion of GSH precedes lipid oxidation and atherogenesis in vivo **(Biswas et al. 2005)**.

Oxidation and inflammation are intertwined processes and it is well established that the pathophysiology of common disease states such as cancer, cardiovascular diseases and neurodegenerative diseases are associated with chronic inflammation **(Cicerale et al. 2012)**. Phenolic compounds derived from EVOO have been reported to have also a significant anti-inflammatory capacity. In vivo and in vitro research has suggested that the dietary intake of EVOO, containing significant concentrations of phenolics, may attenuate inflammatory responses in the body and therefore reduce the risk of chronic inflammatory disease development **(Corona et al. 2009, Khymenets et al. 2009, Konstantinidou et al. 2010)**.

It has been demonstrated in humans that administration of phenol-rich virgin olive oil or particular olive oil phenolics decreased the concentration of different markers of inflammation like arachidonic acid and its metabolites, thromboxane B2 (TXB2) and leukotriene B4 (LTB4) **(Weinbrenner et al. 2004, Visioli et al. 2005, Bogani et al. 2007)**, interleukin 6 (IL-6) and C-reactive protein (CRP) **(Fito et al. 2008)**, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) **(Beauchamp et al. 2005)**.

Olive oil phenolics at nutritionally relevant concentrations also inhibited endothelial adhesion molecule expression **(Carluccio et al. 2003)** and human platelet activity in vitro **(Togna et al. 2003)**.

Blood platelets play a major role in CVD and atherosclerosis, in that, chronic and recurrent damage to the vascular epithelium results in the development of lesions. These lesions then stimulate endothelial adhesion molecules expression, platelet activity, and aggregation **(White 1994, De La Cruz et al. 2000)**.

The ingestion of phenolic compounds from olive oil can also influence the gut microbial balance since most of them are not completely absorbed into the upper parts of the gastrointestinal tract and reach the lower parts where they are metabolised by the gut microflora **(Corona et al. 2006)**.

In addition, phenolics have demonstrated their efficacy in vitro as inhibitors of a wide range of microbial gastrointestinal pathogens such as Escherichia coli **(Medina et al. 2007)** or Helicobacter pylori **(Romero et al. 2007)**, respiratory pathogens such as Haemophilus influenzae **(Bisignano et al. 1999)** or Mycoplasma pneumoniae **(Furneri et al. 2004)** dental pathogens like Streptococcus mutans **(Medina et al. 2006)** and genital pathogens such as Candida albicans **(Medina et al. 2006)** or even virus like herpes mononucleosis **(Fredrickson 2000)** and the para-influenza type 3 virus **(Ma et al. 2001)**.

1.8.1 Phenolic extracts

Nowadays, it is clear that many of the beneficial effects of ingesting EVOO are due to its minor polyphenol compounds such as flavonoids, lignans (acetoxypinoresinol), secoiridoids (oleuropein-aglycone and ligstroside aglycone) and their hydrolysis and metabolic products HT and TYR, HVA respectively, among others. Most of studies focus mainly on the activity of the individual compounds, but recently there is great interest to evaluate the action of phenolic extracts (PE). In fact, at cellular level, the activity of the total phenolic fraction extracted from EVOO and the plausible action mechanisms have not been completely described.

A recent study showed the anti-oxidant and cytoprotection of an EVOO PE in Caco-2 cell exposed to a mycotoxin. The extract protected epithelial cells significantly decreasing ROS production and promoting cell viability and proliferation **(Chiesi et al. 2015)**.

The effect of a PE from olive oil was also analysed on the antioxidant status in rats by DPPH and FRAP in plasma and by superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) activities in erythrocytes. Plasma non-enzymatic antioxidant capacity was significantly modulated (increased DPPH and FRAP values) by the PE, that was also able to significantly decrease SOD and GPX and increase CAT activities **(Rubio et al. 2014)**.

PE down regulate also inflammatory responses in different districts.

A study conducted in LPS-stimulated murine macrophages established the anti-inflammatory activity of an EVOO PE: in particular it decreased nitric oxide (NO) and ROS generation, induced a significant down-regulation of inducible nitric oxide synthase (iNOS), COX-2 and microsomal prostaglandin E synthase-1 (mPGES-1) protein expressions, in addition reduced mitogen-activated protein kinase (MAPK) phosphorylation and prevented the nuclear transcription factor kappa B (NF-KB) translocation to the nucleus **(Cardeno et al. 2014b)**.

The protective effect of dietary PE was also analysed in the inflammatory response associated to chronic colitis model in mouse. In animals treated with the extract disease activity index (DAI) and cell proliferation were significantly reduced, as well as MCP-1, TNF-α, COX-2 and iNOS expression levels. In addition, notably down-regulated JNK phosphorylation, prevented IκBα (inhibitory) degradation and peroxisome proliferator activated receptor gamma (PPARγ) deactivation **(Sanchez-Fidalgo et al. 2013)**.

In another study the effects of EVOO PE were evaluated in a model of rheumatoid arthritis, the collagen-induced arthritis model in mice. The study demonstrated that PE decreases joint edema, cell migration, cartilage degradation and bone erosion. PE significantly reduced the levels of pro-inflammatory cytokines and prostaglandin E2 in the joint as well as the expression of COX-2 and mPGES-1. PE inhibited also c-Jun N-terminal kinase, p38 and signal transducer and activator of transcription-3. In addition, PE decreases NF-KB translocation leading to the down-regulation of the arthritic process **(Rosillo et al. 2014)**.

Different studies have also demonstrated that PEs possess anticarcinogenic properties.

An *in vitro* study showed for example that two EVOO PEs have an antiproliferative effect on colon cancer cells through the interaction with estrogen-dependent signals involved in tumor cell growth **(Pampaloni et al. 2014)**.

Other studies demonstrated the preventive effect of a complex mixture of PE against oxidative DNA damage in human peripheral blood mononuclear cells (PBMCs), promyelocytic leukemia (HL60) **(Fabiani et al. 2008)** and HeLa cells **(Erol et al. 2012)**

1.8.2 Hydroxytyrosol, tyrosol and homovanillic alcohol

Hydroxytyrosol (Figure 4) and tyrosol (Figure 5) are the main phenolic alcohols of EVOO **(Gomez-Alonso et al. 2002)**. Their concentration is low in fresh oils, but increases during oil storage due to the hydrolysis of EVOO secoiridoids that contain them in their molecular structures (oleuropein and ligstroside) **(Brenes et al. 2001)**. The homovanillic alcohol (Figure 6) is the methylated derivative and the major identified metabolite of HT in humans, product of catechol-O-methyltransferase (COMT) activity, and also naturally present in EVOO **(Manna et al. 2000, Tuck et al. 2002)**.

Olive oil phenol alcohols seem to be quite similar in molecule size, although do not exhibit close similarity in their structures: HT is a catechol (o-diphenol), TYR is a phenol and HVA is a methoxyphenol. The configuration of compounds is expected to moderate penetration into membranes and thus affect antioxidant performance in biological systems **(Paiva-Martins et al. 2003)**.

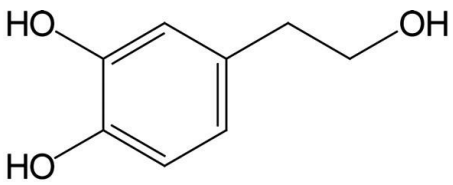


Figure 4: Hydroxytyrosol

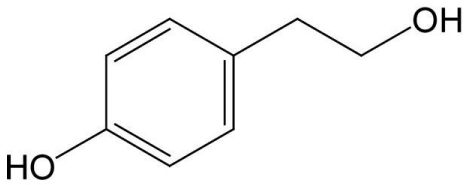


Figure 5: Tyrosol

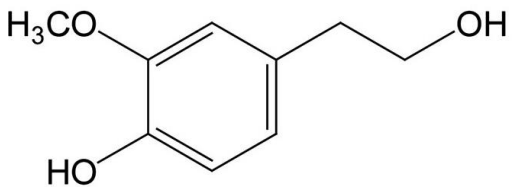


Figure 6: Homovanillic alcohol

It is well-known that the antioxidant properties of o-diphenols are related to hydrogen-donation, i.e. their ability to improve radical stability by forming an intramolecular hydrogen bond between the free hydrogens of their hydroxyl group and their phenoxyl radicals **(Visioli et al. 2002)**. In fact, although specific investigations on the structure-activity relationship of olive oil phenols are yet to be carried out, similar studies have been performed on flavonoids and have indicated that the degree of antioxidant activity is correlated with the number of hydroxyl substitutions **(Rice-Evans et al. 1996)**.

The results of different studies showed that HT and its derivatives (all sharing o-diphenolic structure) are the most potent radical scavengers of olive oil exercising a high antioxidant capacity, whereas monophenols as TYR (with single hydroxyl substitutions) are quite weak, with a low activity. Few and controversial data are available on the radical scavenging activities of HVA, but several studies have shown to be partially active against reactive species in several detection systems **(Tuck et al. 2002, Grasso et al. 2007)**.

HT displayed its effective antioxidant characteristics, scavenging free radicals, chelating transition metals (Cu^{2+} and Fe^{3+}), breaking peroxidative chain reactions, preventing lipid peroxidation, inhibiting hypochlorous acid derived radicals **(Hu et al. 2014)**.

TYR, although has been reported to have a low antioxidant capacity as compared to HT, has scavenging effects on peroxynitrite **(De la Puerta et al. 2001)** and O_2^- **(Bertelli et al. 2002)** and HVA can actively reduce transition metals **(Aguiar et al. 2007)**.

Although the bioactions of phenolic compounds are commonly related to their free radical scavenging activity, current evidence strongly supports that HT and TYR may also provide an indirect protection through increasing the endogenous defense systems involving higher expressions of certain enzymes with antioxidant activities **(Jemai et al. 2008a)**.

Several studies have demonstrated the benefits of phenolic compounds against oxidative damage of macromolecules, in particular lipids (LDL) and DNA. Recently, the European Food Safety Authority (EFSA) has recognized protective effects of the olive oil phenolic compounds on LDL oxidation, in particular of HT **(EFSAJournal 2011)**, already widely showed in several *in vitro* and *in vivo* studies **(Salami et al. 1995, Marrugat et al. 2004, Gonzalez-Santiago et al. 2006)**. The antioxidant capacity of phenolics on LDL has been suspected for decades. In fact, in 1995 Salami et al. **(Salami et al. 1995)** reported lower levels of isoprostanes and thiobarbituric acid-reactive substances, and therefore lipid peroxidation, in LDL due to HT. Also TYR has been shown to be effective in inhibiting LDL-cholesterol oxidation, and preventing the modification of the apoproteic moiety **(Caruso et al. 1999)**. Subsequent studies confirmed the protective effect of these antioxidants on LDL lipoproteins, suggesting a modulation of atherosclerosis, working with in vitro models as well as with animals **(Marrugat et al. 2004, Gonzalez-Santiago et al. 2006, Visioli et al. 2006, Di Benedetto et al. 2007)**. It has also been confirmed that the incubation of plasma with phenolic compounds of olive oil (among them HT) reduces and prevents LDL oxidation **(Leenen et al. 2002)**. More recently, it has been demonstrated that olive phenols and their metabolites are much more efficient inhibitors of lipid and protein oxidations compared to vitamins C and E **(Roche et al. 2009)**

In addition, Jemai et al. demonstrated that in rats fed with a cholesterol-rich diet, HT was able to promote hypocholesterolemia, lowering LDL plasma levels and total cholesterol; also, they increased the levels of HDL and the activity of antioxidant enzymes reducing LDL oxidation **(Jemai et al. 2008a, Jemai et al. 2008b)**.

Intercellular and vascular cell adhesion molecules (ICAM-1, VCAM-1 and E-selectin) are crucial for endothelial activation an early step in atherogenesis; HT and HVA were able to inhibit endothelial activation reducing cell surface expression of all the adhesion molecules, inhibiting their mRNA levels, thus

decreasing monocyte adhesion to endothelial cells **(Carluccio et al. 2003, Dell'Agli et al. 2006)**.

Mixtures of olive phenols were able to reduce hydrogen peroxide (H_2O_2)-induced DNA damage in cells **(Nousis et al. 2005, Fabiani et al. 2008)** as well as individual olive oil phenols as HT and to a lesser extent HVA **(Quiles et al. 2002, Nousis et al. 2005, Grasso et al. 2007, Fabiani et al. 2008)**. The activity of TYR against hydroperoxyde induced DNA damage remains unclear: it is able to reduce DNA oxidation only at high doses in oxidative-stress-sensitive cells **(Quiles et al. 2002)**, and did not exert any protection activity in hydrogen peroxide exposed Jurkat cells **(Nousis et al. 2005)**, nevertheless, in a study with activated monocytes it was reported to be more effective than HT **(Fabiani et al. 2008)**. HT was also highly protective against the peroxynitrite dependent nitration of tyrosine and DNA damage in vitro **(Deiana et al. 1999)**. The role of phenolic compounds on in vivo DNA oxidative damage after olive oil consumption in humans remains unclear. The protective role was observed against DNA oxidative damage taking place in peripheral blood mononuclear cells or lymphocytes **(Yamada et al. 1991, Salvini et al. 2006)**, but not on the whole body DNA oxidation **(Hillestrom et al. 2006, Machowetz et al. 2007)** measured by accumulation of DNA oxidative products in urine, where it was lowered irrespectively to amount of phenols by any type of olive oil **(Machowetz et al. 2007)**.

Additional evidence of the antioxidant properties of HT was provided by Manna et al. **(Manna et al. 1997)**, who demonstrated an antioxidant effect of HT (but not of TYR) in a model of oxidative stress induced in intestinal epithelial cells. In this experimental model TYR, which lacks the ortho-diphenolic structure, was found to be ineffective. The same group described a protective effect of HT toward hydrogen peroxide-induced damage to human erythrocytes **(Manna et al. 1999)**.

TYR has been effective protecting the Caco-2 intestinal mucosa cells against the cytostatic effects produced by oxidized LDL **(Giovannini et al. 1999)** and inhibiting the peroxynitrite-induced neuronal oxidative damage in cultured

neurons **(Vauzour et al. 2007)**. TYR has also been shown to improve the intracellular antioxidant defence systems in macrophage-like cell line **(Di Benedetto et al. 2007)**.

Deiana et al. investigated the capacity of HT, TYR and HVA to inhibit H_2O_2 induced oxidative damage in LLC-PK1 studying the effect on specific cell membrane lipid targets, unsaturated fatty acids and cholesterol. Both HT and HVA were able to prevent in renal cells the lipid peroxidation process that plays a central role in tubular cell injury. TYR, instead, in this experimental model, did not exert any protective effect **(Deiana et al. 2008, Loru et al. 2009)**. It was also demonstrated that both compounds (HT and HVA) were capable of inhibiting hydrogen peroxide-induced kidney cell injury via an ability to interact with both MAP kinase and PI3 kinase signalling pathways, albeit at different concentrations **(Incani et al. 2010)**.

The same research group investigated the capacity of HT, TYR and HVA to inhibit the insult of dietary lipid hydroperoxides (tert-butyl hydroperoxide-TBH) on the intestinal mucosa, using cultures of Caco-2 and studying the effect of treatment on specific cell membrane lipid targets. Pre-treatment with these simple phenols protected Caco-2 cells from oxidative damage **(Deiana et al. 2010)**.

Olive oil phenolics have also shown to display anti-inflammatory properties. It has been observed that HT inhibits in vitro platelet aggregation induced by TXB2 production and collagen **(Petroni et al. 1995)**. The same effect was observed in healthy rats assigned to diet supplemented with HT **(Gonzalez-Correa et al. 2008)**: in this study it was proposed that both an inhibition of COX-2 with a related decrease of TXA2 blood levels and an increase of vascular nitric oxide production may contribute to this effect **(Gonzalez-Correa et al. 2008)**.

TYR has been shown to inhibit the production of LTB4 in rat peritoneal leukocytes, while similar effects have been seen in human platelet cells and leukocytes **(Petroni et al. 1995, Petroni et al. 1997, de la Puerta et al. 1999).** An important observation is that the capacity of TYR to prevent H_2O_2 production by

macrophages induced by oxidized LDL, arachadonic acid release, and PGE2 synthesis, was enhanced by the addition of β-sitosertinol **(Vivancos et al. 2008)**.

In a study using J774 murine macrophage cells treated with LPS, a potent macrophage activating stimulus, treatment with HT resulted in the inhibition of expression of two pro-inflammatory genes, iNOS and COX-2. This was found to be due to the prevention of activation of the transcription factors NF-κB, STAT-1α, and IRF-1 **(Maiuri et al. 2005)**. HT has been reported to possess significant anti-inflammatory actions in an animal model of inflammation and attenuate TNFα and IL-1β expression, which are pro-inflammatory cytokines often observed in inflammatory diseases **(Gong et al. 2009)**. Furthermore, an in vitro study has reported the attenuation of iNOS, COX-2 and TNFα by HT in LPS- challenged human monocytic THP-1 cells **(Zhang et al. 2009)**.

EVOO rich in HT diets exerted a noteworthy beneficial effect in chronic dextran sodium sulfate (DSS)-induced colitis by cytokine modulation and COX-2 and iNOS reduction via downregulation of p38 MAPK. In addition to the beneficial effect of EVOO, supplementation of the diet with HT may improve chronic colitis through iNOS downregulation plus its antioxidant capacity **(Sanchez-Fidalgo et al. 2012)**.

HT, at nutritionally relevant concentrations, reduced also matrix metalloproteinase MMP-9 and COX-2 induction in activated human monocytes via protein kinase C (PKCα, PKCβ1) inhibition **(Scoditti et al. 2014)**.

A long period of oxidative stress and inflammatory responses will cause many diseases. A pro-inflammatory cytokine plays an important role in the pathogenesis of chronic and autoimmune diseases. Some data shows that the intake of polyphenols such as OL and HT can affect the formation of advanced glycation end products and their interaction with respective receptors, and modulate chronic inflammatory diseases, for example, type II diabetes, rheumatoid arthritis, and inflammatory bowel disease **(Kawaguchi et al. 2011)**.

2. Aim of the study

A healthy and proper diet is essential to prevent the onset of various diseases, as with the diet we can also introduce harmful substances.

Among others, the oxysterols are particularly relevant; they can be absorbed from the diet and are the oxidized products of cholesterol present in cholesterol-containing foodstuffs after cooking, processing and storage, such as meat, fish, dairy products and eggs. They can be also generated endogenously from either the spontaneous or enzymatic oxidation of cholesterol.

Oxysterols have been shown to exert several *in vitro* and *in vivo* biochemical activities, physiological but mostly pathological. In particular, they may induce cytotoxicity, cell death (necrosis and apoptosis), pro-oxidant and pro-inflammatory effects in different cells and tissues and have been linked with the onset and development of major chronic diseases.

Recently it has been shown that they are involved in irreversible damages to the colonic epithelial layer, because of their ability to induce oxidative stress, apoptosis and inflammatory reactions, and they have been associated with the pathogenesis of the IBD and with an increased risk of developing colon-rectal cancer.

The intestinal epithelium sits in fact at the interface between an organism and its luminal environments and represents the first barrier of defence. As such it is exposed to a wide range of oxidant agents, either endogenous or derived from the diet, like oxysterols, that can damage the intestinal integrity causing various intestinal diseases.

Substantial evidence shows that oxysterols contribute also to the development of atherosclerosis as they are common components of oxidized-LDL and have been detected in both human plasma and in the core of fibrotic plaques inducing and sustaining chronic inflammatory process within the vascular well, especially in hypercholesterolemic subjects.

Over the years, the so-called Mediterranean diet has become widely associated with improved health and well-being as well as protection against chronic

diseases. Epidemiological and clinical studies have demonstrated that low chronic disease risk observed in Mediterranean areas seems to be ascribed to high intake of extra virgin olive oil, in particular for the anti-oxidant and anti-inflammatory properties of the phenolic fraction.

The phenolic fraction of extra virgin olive oil contains several kinds of chemical compounds, mainly complex phenols, like secoiridoids derivatives, that are not directly absorbed, but undergo gastrointestinal biotransformation, increasing the relative amount of hydroxytyrosol and tyrosol entering the small and large intestine where can be further metabolised before absorption. The phenolic compounds which are absorbed don't reach relevant blood concentrations, however they have been shown to be sufficient to exert a biological systemic effect. Most of the phenols remains in the intestine, where their concentration of these simple phenols may be in the high μM and they might play an important role in preventing oxidative stress linked diseases.

The aim of this study was to investigate the protective effect of the oil polyphenols (as a whole extract or as pure compounds) against the harmful action of oxysterols both at intestinal and systemic level.

The first part of this study focused on the capability of extra virgin olive oil phenolic extracts to counteract the pro-oxidant, pro-apoptotic and pro-inflammatory effects induced by dietary oxysterols in the gut.

Caco-2 cell line, an accepted model for human intestinal epithelial cells, was used to investigate these effects in the gut. Caco-2 are an adenocarcinoma colon cell line that spontaneously undergo differentiation into enterocyte-like cells at about 21 days after plating. Differentiated Caco-2 cells express microvilli, basolateral membranes separated by tight junctions, intestine-specific antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione reductase (GRED) and catalase, and possess absorptive properties similar to those of normal intestine.

The oxidative damage was induced by an oxysterols mixture composed by the most widely represented oxysterols in processes or/and stored cholesterol-rich foods: 7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, cholesterol 5α, 6α-epoxide and cholesterol 5β, 6β-epoxide. The phenolic extracts used, were obtained from monovarietal extra virgin olive oils of two olive cultivars among the most common and widespread varieties in Sardinia (Italy), Bosana and Nera of Gonnos.

Changes in the cellular redox state were evaluated determining ROS generation, variation of intracellular GSH concentration and GPX activity; malondialdehyde (MDA) production, a secondary product of lipid peroxidation, was then evaluated as marker of oxidative damage together with induction of cell death, analysed through a viability test and measuring the activity of caspase 3, as apoptotic index.

Caco-2 were also used in order to investigate the production of pro-inflammatory cytokines, specifically IL-6 and IL-8 already known to be crucial in chronic intestinal inflammation and sustaining intestinal barrier disruption.

It is also well known that polyphenols can act by modulating different cell signaling pathways and, through these, exert their beneficial effects.

For this reason another purpose of this study was to analyse the signaling pathways modulated by oxysterols and olive oil phenolic extracts to clarify the molecular mechanism involved in the processes of survival/apoptosis and in the antioxidant and anti-inflammatory activity (Akt/PKB, MAPK).

The second part of this study focused on the protective action of pure olive oil phenolics, which can be found in the blood stream after absorption, hydroxytyrosol, tyrosol, and homovanillic alcohol, against the pro-oxidant and pro-inflammatory activity of oxysterols at systemic level.

Peripheral blood mononuclear cells (PBMCs) were used for this purpose; these are composed by any blood cell having a round nucleus such as lymphocytes, monocytes and macrophages, and were separated from whole blood of healthy volunteers.

A different biologically compatible oxysterols mixture was used, composed by the most widely represented oxysterols in the blood of hypercholesterolemic subjects, derived in part from the diet and in part from oxidation of endogenous cholesterol: 7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, cholesterol 5α, 6α-epoxide, cholesterol 5β, 6β-epoxide, cholestane-3β, 5α, 6β-triol and 25-hydroxycholesterol.

It is well known that oxysterols are able, directly or through a ROS-mediated mechanism, to enhance pro-inflammatory cytokine secretion in human macrophages or monocytes, by also modulating redox-sensitive pathways.

Therefore PBMCs were used in order to analyse the ability of the olive oil simple phenols to inhibit the increase of ROS and pro-inflammatory cytokine/chemokine production (MCP-1, IL-1β, MIF and RANTES) induced by the oxysterols mixture and to modulate the signaling pathways (MAPK) involved in these processes.

3. Materials and methods

3.1 Reagents and chemicals

All solvents, of analytical grade, were purchased from Merk (Darmstadt, Germany).

7α-hydroxycholesterol (cholest-5-en-3β,7α-diol), 7β-hydroxycholesterol (cholest-5-en-3β,7β-diol) and cholestane-3β,5α,6β-triol (5α,6β-dihydroxycholestanol) were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). 7-ketocholesterol (3β-hydroxy-5-cholestene-7-one), 25-hydroxycholesterol (cholest-5-ene-3β,25-diol), cholesterol 5α,6α-epoxide (5α,6α-epoxycholestan-3β-ol), cholesterol 5β,6β-epoxide (5β,6β-epoxycholestan-3β-ol), Neutral red, Trypan blue, 2-thiobarbituric acid (TBA), trichloroacetic acid (TCA), 2-(N-morpholino) ethanesulfonic acid (MES), meta-phosphoric acid (MPA), tris (hydroxymethyl) aminomethanehydrochloride (tris-HCL), ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis (2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), sodium chloride (NaCl), Triton X-100, protease inhibitor cocktail, phenylmethanesulfonyl fluoride (PMSF), sodium orthovanadate, sodium pyrophosphate, sodium fluoride, DL-dithiothreitol (DTT), 2′,7′-Dichlorofluorescein diacetate (DCFH-DA), horseradish peroxidase-conjugated goat anti-rabbit secondary antibody, and Bradford reagent, were purchased from Sigma Aldrich (Italy, UK). 1,1,3,3-Tetraethoxypropane (TEP) were purchased from Fluka (AG, Switzerland). The kits to analyse gluthatione peroxidase "*Glutathione Peroxydase Assay kit*" and gluthatione "*Glutathione Assay kit*" were purchased from Cayman Chemical Company (Ann Arbor, USA). The kits to analyse interleukin 6 and 8 production "*Human IL-6 ELISA Kit"* and "*Human IL-8 ELISA Kit"* were purchased from Gen-Probe (France). The primary antibodies anti-phospho-JNK 1/2 anti-JNK, anti-phospho-p38, anti-p38, anti-phospho-Akt (Ser473), anti Akt, anti-phospho-MAP Kinase1/2 (ERK 1/2), anti-MAP Kinase 1/2 (ERK 1/2), cleaved caspase 3, β-actin and the enhanced chemiluminescence (ECL) reagent were purchase from Millipore (Watford, UK). Gels and all material for electrophoresis and immunoblotting were purchased from Invitrogen (Milan,

Italy). Nitrocellulose membranes were obtained from Amersham (Little Chalfont, UK).

Hydroxytyrosol (HT), Tyrosol (TYR) and Homovanillic Alchool (HVA) were obtained from Cayman Chemical (Ann Arbor, USA). The Vacutainer, sodium-heparin coated tubes, were purchased from Greiner Bio-one Ltd (Gloucestershire, UK).

The kit for the preliminary cytokines analysis "*Human Cytokine Array Panel A*" and the kits to analyse IL-1β "*Human IL-1β/IL-1F2 DuoSet*", RANTES "*Human CCL5/RANTES DuoSet* " and MIF "*Human MIF DuoSet*" were obtained from R&D systems (Abingdon, UK). The kit to analyse MCP-1 "*Human CCL2 (MCP-1) ELISA Ready-SET-Go!*"was purchased from eBioscience (Hatfield, UK).

3.2 Extra virgin olive oils (EVOO)

Monovarietal extra virgin olive oils, were obtained from olives of the Bosana and Nera of Gonnos cultivars harvested and processed by AGRIS, (Villasor, Cagliari), in the years 2012 and 2013. The oils were stored in 5 l steel tanks at 4°C until analysis.

3.3 Extraction of EVOO phenols and secoiridoids

A litre of oil was divided into 4 fractions of 250 ml. Each fraction was put in a 500 ml separating funnel and extracted by vigorous shaking with 100 mL of MeOH/H_2O 80:20 (v/v). After 20 min incubation, the oil phase was separated from the aqueous one, put in a round-bottom flask and concentrated in a Rotavapor (30°C, minimum pressure 30 mbar). The oil phase was extracted again with 100 ml of MeOH/H_2O 80:20, repeating the procedures previously described. The extraction of the oil phase was repeated two more times for a total of four extractions on each 250 ml oil fraction. The aqueous phases concentrated in the round-bottom flask were put in a separating funnel with 100 ml of isohexane. After shaking, the concentrated aqueous phase was collected and dried in a Rotavapor. The residue was dissolved in MeOH, filtered through a cellulose acetate syringe Whatman GD/X 0.45 nm 25 mm and dried in a Rotavapor.

3.4 Determination of phenolic compounds and secoiridoids by liquid chromatography and diode array detector (LC-DAD)

The quali/quantitative determination of phenolic compounds and secoiridoids contained in the hydrophilic fraction was performed using a LC-DAD method **(Sarolic et al. 2014)**. A Varian Pro Star HPLC system equipped with a pump module 230, was used together with an 410 autosampler module (injection volume 10 μl) and a diode array detector UV SpectroSystem 6000lp (ThermoSeparation, San Jose, CA, USA). The separation was obtained with a Gemini C18 column (150 x 4.60 mm, 3 μm, Phenomenex, Casalecchio di Reno, BO, Italy) using a mixture of 0.2 M H_3PO_4 (solvent A) and acetonitrile (solvent B) at a constant flow of 1.0 ml/min and mixed in a linear gradient as follows: t = 0, A:B 85:15, v/v; 60:40 (v/v) in 30 min; 40:60 (v/v) in 10 min and finally 100% B until 50 min. Before each injection, the LC system was stabilized for 10 minutes with the initial ratio A:B (85:15, v/v). The analysis of phenols was performed at optimal wavelengths of detection: 280 nm (hydroxytyrosol, tyrosol, vanillic acid, oleuropein derivatives and ligstroside), 313 nm (p-coumaric acid) and 360 nm (luteolin and apigenin). The derivatives of oleuropein and ligstroside were identified comparing literature data **(Garcia-Villalba et al. 2010, Dierkes et al. 2012)**. Chromatograms and spectra were processed with the software ChromQuest 2:51 V (ThermoQuest, Rodano, MI, Italy). The standard solutions were prepared using methanol and the working solutions using a mixture of methanol:water (80:20, v/v). Calibration curves were constructed using external standards, correlating peak area with concentration (correlation values ranged between 0.9993 and 0.9999). All compounds were assayed using the calibration curve obtained with the respective standard, except the derivatives of oleuropein and ligstroside that were assayed using the calibration curve of oleuropein.

The chemical composition of the EVOO phenolic extracts used (Bosana and Nera of Gonnos) is reported in Table 1.

Compound	Bosana (mg/g) mean ± sd	Nera of Gonnos (mg/g) mean ± sd
hydroxytyrosol	10,7 ± 0,1	5,2 ± 0,2
tyrosol	19,0 ± 0,7	7,0 ± 0,3
vanillic acid	0,8 ± 0,0	0,5 ± 0,0
p-coumaric acid	1,4 ± 0,0	0,4 ± 0,0
3,4-DHPEA-EDA	27,9 ± 1,1	7,1 ± 0,2
pinoresinol	9,0 ± 0,3	2,7 ± 0,1
p-HPEA-EDA	19,6 ± 0,8	17,0 ± 0,9
luteolin	3,5 ± 0,1	3,9 ± 0,1
acetoxypinoresinol	3,2 ± 0,1	0,6 ± 0,0
3,4-DHPEA-EA	30,8 ± 1,5	24,9 ± 1,3
apigenin	0,9 ± 0,0	1,7 ± 0,0
p-HPEA-EA	15,4 ± 1,7	18,6 ± 0,9

Table 1: Chemical composition and concentration of single compounds expressed as mg/g, of Bosana and Nera of Gonnos phenolic extracts.
(3,4-DHPEA-EDA, decarboxymethyl oleuropein aglycone; p-HPEA-EDA, decarboxymethyl ligstroside aglycone; 3,4-DHPEA-EA, oleuropein aglycone; p-HPEA-EA, ligstroside aglycone).

3.5 Caco-2 cell cultures

3.5.1 Material for cell cultures

The Caco-2 cell line (human epithelial colorectal adenocarcinoma cells) was obtained from ECACC, (Salisbury, Wiltshire, United Kingdom).The medium "*Dulbecco's modified eagle medium"*(D-MEM), the heat-inactivated fetal bovine serum (FBS), the penicillin-streptomycin solution, the phosphate-buffered saline (PBS) and the tryspin-EDTA were purchased from Invitrogen (Milan, Italy).

3.5.2 Maintenance of cell cultures

Caco-2 cells were maintained in T75 flasks until their confluence reached the 80%, in D-MEM supplemented with 10% FBS, 1% antibiotic/antimycotic solution (100 U/ml penicillin, 100 mg/ml streptomycin) at 37°C in a 5% CO_2 humidified atmosphere. The subcultures were prepared by removing the cells with trypsin solution at 1% and then seeded into 6-24-96 well plates and Petri dishes for different experiments. Cells were cultured for 21 days, replacing the medium thrice weekly, to allow their spontaneous differentiation.

3.5.3 Preparation of the oxysterols mixture

The mixture of the most widely represented dietary oxysterols was prepared using pure standard compounds diluted in ethanol.

The oxysterols and their relative percentage used in the mixture were: 7α-hydroxycholesterol (4.26%), 7β-hydroxycholesterol (14.71%), 7-ketocholesterol (42.96%), 5α,6α-epoxycholesterol (32.3%), 5β,6β-epoxycholesterol (5.76%) **(Plat et al. 2005)**. The concentration of the oxysterols mixture was calculated using an average molecular weight of 403 g/mol **(Biasi et al. 2009)**.

3.5.4 Measurement of intracellular reactive oxygen species (ROS) production

ROS generation in Caco-2 cells was determined using the fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA).

When applied to intact cells, the non-ionic, non-polar DCFH-DA crosses cell membranes and is hydrolyzed enzymatically by intracellular esterases to non-fluorescent DCFH. In the presence of reactive oxygen species (ROS), DCFH is oxidized to highly fluorescent dichlorofluorescein (DCF).

To quantify ROS production, differentiated Caco-2 cells were seeded in 96-well plates. The old medium was removed, cells were washed with 200 µl of PBS and incubated for 30 minutes with DCFH-DA 10 µM. Then the DCFH-DA was removed and cells were added with oxysterols mixture (60-90-150 µM) for different incubation times (5-180 min).

To assess the protection by the two phenolic extracts, cells, after treatment with DCFH-DA, were incubated simultaneously with oxysterols mixture 150 µM and phenolic extracts at different concentrations (5, 10, 25 µg/ml) for 1h.

ROS production was monitored by reading the fluorescence emitted from the DCF **(Dinicola et al. 2013)**, taking readings at intervals of 5 min for 180 minutes (for the first set of experiments) and reading after 1 h of incubation (for the second one), using a micro plate reader at controlled temperature of 37°C (Plate reader, Infinite 200, GENios TECAN). The reading was performed using an excitation wavelength of 490 nm and an emission wavelength of 520 nm. The fluorescence values were expressed as f/f0, where f is the fluorescence of the samples pre-treated with DCFH-DA and treated with the compounds and f0 the fluorescence of samples not pre-treated with DCFH-DA.

3.5.5 Quantification of intracellular glutathione (GSH)

In order to quantify the level of GSH a commercial kit ("*Glutathione Assay kit*" Cayman Chemical Company) based on an enzymatic recycling method was used. The sulfhydryl group of GSH reacts with DTNB (5,5'-dithio-bis-2-nitrobenzoic acid) and produces a yellow colored 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide, GSTNB (between GSH and TNB) that is concomitantly produced, is reduced by glutathione reductase to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to this recycling reaction which in turn is directly proportional to the concentration of GSH in the sample.

Caco-2 cells were cultured in Petri dishes 9 cm in diameter and used after 20 days of differentiation. In a first set of experiments cells were treated with the oxysterols mixture 150 μM for different times (30 minutes-24 h); in another set of experiments, cells were pre-treated 30 minutes with increasing concentration of phenolic extracts (5-25 μg/ml) and then treated with the oxysterols mixture 150 μM in complete medium with 2,5 % of FBS for 30 minutes. At the end of incubation time, the medium was removed, the cells washed with cold PBS and then scraped with 1 ml of PBS and centrifuged at 1700 x g for 10 minutes at 4°C. The obtained cellular pellet was resuspended with a cold buffer prepared mixing 50 mM of MES pH 6-7 and 1 mM of EDTA. The samples were then mixed, sonicated for 10 minutes and centrifuged at 10000 x g for 15 minutes at 4°C. 600 μl of supernatant were added with 600 μl of MPA reagent to deproteinate the sample. Since almost all biological samples used for GSH measurement contain large amounts of proteins, it is necessary to remove as much protein as possible from the sample to avoid interferences due to particulates and sulfhydryl groups on proteins in the assay. Then, the supernatant obtained after centrifugation was used to analyse the GSH with the commercial kit the following manufacturer's instructions. The measurement of the absorbance of TNB at 405 or 412 nm provides an accurate estimation of GSH in the sample.

3.5.6 Evaluation of glutathione peroxidase activity (GPX)

The changes in GPX activity were assessed using a commercial kit ("*Glutathione Peroxydase Assay kit*" Cayman Chemical Company), based on spectrophotometric determination of $NADP^+$, generated in the reaction mixture according to the reactions shown below

$$ROOH + 2GSH \xrightarrow{GPX} ROH + GSSG + H_2O$$

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+$$

To perform the assay, cells were cultured in 6-well plates and treated after 20 days of differentiation. In a first set of experiments cells were treated with the oxysterols mixture 150 μM for different times (6-36 h), and in another set Caco-2 cells were pre-treated for 30 min with increasing concentration of phenolic extracts (5-25 μg/ml) and then treated with the oxysterols mixture 150 μM in complete medium with 2,5 % of FBS for 18 h.

At the end of incubation time, the medium was removed, the cells washed with PBS and then scraped with 1 ml of cold PBS and centrifuged at 1700 g for 10 minutes at 4°C. The obtained cellular pellet was resuspended with a cold buffer prepared mixing Tris-HCl (50 mM), EDTA (5 mM) and DTT (1 mM). The samples were then mixed and centrifuged at 10000 x g for 15 minutes at 4°C. The obtained supernatant was collected and used to analyze the enzymatic activity with the commercial kit following the manufacturer's instructions.

The absorbance was spectrophotometrically measured at a wavelength of 340 nm using a plate reader.

3.5.7 Evaluation of cell viability/malondialdehyde (MDA) production

To evaluate the cytotoxicity and the MDA production induced by the oxysterols mixture and the protective effects of the phenolic extracts, cells were cultured into 24-well plates with complete medium until differentiation.

In the first set of treatments, the medium was replaced with new complete medium at 2,5% of FBS and the cells were incubated for 24 hours at 37°C with increasing concentration (30-150 μM) of oxysterols mixture.

In the second set of experiments, cells were incubated for 30 min at 37°C with different concentrations (1-25 μg/ml) of phenolic extracts and then added with the oxysterols mixture 150 μM for 24 h.

After that, for both types of treatment, the medium was collected for MDA analysis using the TBARS test, and the cellular viability was evaluated using the Neutral red test.

3.5.7.1 TBARS method and HPLC analysis

MDA concentration was determined as MDA-TBA adduct by HPLC **(Templar et al. 1999)**. 400 μl of the supernatants, taken at the end of the incubation time with oxysterols and phenolic extracts, were added with 100 μl of TCA (10%), mixed and incubated at room temperature for 20 minutes, and then added with 200 μl of TBA (0.6%) and incubated at 90°C for 45 min. After centrifugation at 5000 x g for 15 min, aliquots of the supernatant were injected into the HPLC-DAD system System (HPLC-DAD 1050 series, Hewlett Packard). Separation of the MDA-TBA adduct was carried out using a C-8 reversed-phase column (Zorbax Eclipse XDB-C8, 150×4.6 mm, 5μm), with a mobile phase of 50 mM KH_2PO/MeOH (65/35, v/v) at flow rate of 1 ml/min. Detection of the adduct peak was carried out at 532 nm.

For each experiment a calibration curve was prepared using a TEP solution in PBS (0.05–10 μM).

3.5.7.2 Neutral red test

The neutral red method, as originally developed by Borenfreund and Puerner, **(Borenfreund et al. 1984)** is simple, accurate and yields reproducible results. The key component is the vital dye, neutral red (Basic Red 5, Toluylene Red). Viable cells will take up the dye by active transport and incorporate it into lysosomes, whereas non-viable cells will not take up it. The incorporation of the dye into cells depends on their number and on their physiological state.

After treatments with the oxysterols mixture and the phenolic extracts, cells were incubated for 15 minutes at 37°C with Neutral red solution 0,33% in DMEM, in an amount equal to 10% of the culture medium volume (50 μl in 500 μl of medium). At the end of the incubation period, the medium was carefully removed and the cells washed in PBS; the incorporated dye was then solubilized in 1 ml of solubilization solution (CH_3COOH 1%, H_2O 49% and EtOH 50%). The absorbance was spectrophotometrically measured at a wavelength of 540 nm using a plate reader.

3.5.8 Modulation of intracellular signaling pathways

3.5.8.1 Cells treatment

Caco-2 cell were cultured in 6-well plates and used after 20 days of differentiation. In the first set of experiments the cells were incubated for 30 minutes (Akt/PKB, MAPK) and 24 h (Caspase3) with different amounts of the oxysterol mixture (30-150 μM). In the second set of experiments cells were pre-treated with the phenolic extracts (1-5-10 μg/ml) for 30 min and then treated with the oxysterols mixture 60 μM, the lowest concentration that induces cell death, for 30 minutes and 24 h.

3.5.8.2 Extraction and quantification of proteins

At the end of the incubation time, cells were washed with cold PBS and suspended in 200 μl of a cold lysis buffer (Tris base 50 mM, Triton X-100 (1:100 v/v), EDTA 2 mM, EGTA 2 mM, NaCl 150 mM, mammalian protease inhibitor cocktail (1:100 v/v), PMSF 0,5 mM, Sodium ortho-vanadate 1 mM, Sodium pyro-phosphate 5 mM and Sodium fluoride 50 mM) to extract the proteins. The cells were scraped on ice and lysates were collected in eppendorf tubes, incubated on ice for 45 minutes, centrifuged at 15000 x g for 5 minutes and then the supernatants were collected.
The protein concentration was determined by the Bradford protein assay **(Bradford 1976)**.

3.5.8.3 Electrophoresis and immunoblotting

Samples were boiled at 70°C for 10 min in boiling buffer (62.5 mM Tris base, pH 6.8 containing 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.0025% bromophenol blue). The boiled samples were run on 10%, 12% SDS–polyacrylamide gels (20 μg/lane), and the proteins were transferred to nitrocellulose membranes (Hybond-ECL) by semi-dry electroblotting (1.5 mA/cm^2). The nitrocellulose membrane was then incubated in a blocking buffer [TBS supplemented with 0.05% (v/v) Tween 20 (TTBS)] containing 4% (w/v) skimmed milk powder for 1 h at room temperature, followed by three 5 min washes in TTBS. The blots were then incubated with anti-pAkt (Ser473) (1:1000 dilution), anti-Akt (1:1000 dilution), anti-pERK 1/2 (1:1000 dilution), anti-ERK 1/2 (1:1000 dilution), anti-pp38 (1:1000 dilution), anti-p38 (1/1000 dilution), anti-pJNK 1/2 (1:1000 dilution), anti-JNK 1/2 (1:1000 dilution) and Caspase 3 (1:1000 dilution) in TTBS containing 1% (w/v) skimmed milk powder (antibody buffer) overnight at 4°C on a three-dimensional rocking table.

The blots were washed twice for 10 min in TTBS and then incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:2000 dilution) in antibody buffer for 1 h. Finally, the blots were washed twice for 10 min in TTBS, exposed to Hyperfilm-ECL for 2–5 min and developed with the ChemiDoc XRS Imager (BioRad).

The molecular weights of the bands were calculated by comparison with pre-stained molecular weight markers that were run in parallel with the samples. Protein bands were quantified using Image J software.

3.5.9 Evaluation of cytokines production (IL-6 and IL-8) by ELISA

To assess the pro-inflammatory effect induced by the mixture of oxysterols and the ability of olive oil phenolic extracts to prevent this effect, the modulation of two pro-inflammatory cytokine, interleukin-8 and interleukin-6, was evaluated. These cytokines are crucial in chronic intestinal inflammation in sustaining intestinal barrier disruption.

Caco-2 cells were seeded in T 25 flasks with complete medium and used after 20 days of differentiation. Cells were incubated for 30 minutes with the phenolic extracts (25 μg/ml) and then treated for 24 hours with the oxysterols mixture 60 μM. At the end of the treatment, the cell culture medium was collected and centrifuged at 140 x g for 10 minutes and the supernatant was used to quantify the levels of IL-8 and IL-6 by ELISA kit ("*Human IL-8 ELISA Kit"* and "*Human IL-6 ELISA Kit"* Gen-Probe). The absorbance reading was performed using a microplate reader at a wavelength of 450 nm and using as reference the absorbance at a wavelength of 550 nm.

3.6 Peripheral blood mononuclear cells (PBMCs) cultures

3.6.1 Material for cell cultures

The medium RPMI 1640 with HEPES and L-Glutamine and the phosphate-buffered saline (PBS) were purchased from Lonza. The antibiotic solution: pen/strep 5000U/5mg ml and the Histopaque-1077 were purchased from Sigma Aldrich.

3.6.2 PBMCs isolation

Human PBMCs were isolated from freshly collected blood samples obtained from 8 healthy volunteers (male and female), aged between 23 and 40 years. A written consent was obtained from all volunteers, who were enrolled according to specific exclusion and inclusion criteria (Table 2). The trial protocol was reviewed and approved by the Ethics and Research Committee of the University of Reading.

Using standard Ficoll-Paque gradient centrifugation, blood samples, collected in sodium heparin-coated tubes, were transferred to 50 ml tubes and diluted with an equal volume of Histopaque-1077, which is a density gradient separation medium, and centrifuged for 30 minutes at 400 x g. After centrifugation, upper layer (plasma) was kept, and then, the PBMCs layers (the opaque interface) were carefully removed with a pipette, put in new tubes, and resuspended/washed in PBS. This solution was centrifuged first for 10 minutes at 300 x g, then for another 10 minutes at 380 x g and then the supernatant was discarded. Finally, PBMCs were re-suspended in the culture medium consisting of RPMI, 1% v/v glutamine and 1% antibiotics supplemented with autologous plasma (2.5% v/v).

Volunteer inclusion criteria	Volunteer exclusion criteria
Signed informed consent Men and women aged 20 – 40 years Non smokers Good general health	History of drug abuse, including alcohol Participation in experimental trials within 3 months prior to study Use of antibiotics within the previous 3 months Use of prescribed medication Regular use of anti-inflammatory drugs Any kind of inflammatory, auto-immune disease or allergy Any other pathology

Table 2. Volunteer inclusion/exclusion criteria for donating blood

3.6.3 Preparation of the oxysterols mixture

The mixture of the most widely represented oxysterols in hypercholesterolemic subjects and their relative percentage were: 7α-hydroxycholesterol (5%), 7β-hydroxycholesterol (10%), cholesterol α-epoxide (20%), cholesterol β-epoxide (20%), cholestan-3β,5α,6β-triol (9%), 7-ketocholesterol (35%), and 25-hydroxycholesterol (1%). The concentration of the oxysterol mixture was calculated by using an average molecular weight of 403. Ethanol was used as solvent to dilute the oxysterols **(Biasi et al. 2004, Poli et al. 2009)**.

3.6.4 Evaluation of cytokines/chemokines production

3.6.4.1 PBMCs treatments

PBMCs ($1x10^6$ cells/ml) were seeded in 24 well plate in complete RPMI and pre-treated with pure phenolic compounds, hydroxytyrosol, tyrosol and homovanillic alcohol (0.25, 0.5, 1 μM) and incubated for 30 min at 37°C in a 5% CO_2 atmosphere. Then the oxysterols mixture 20 μM was added in the medium for 24 h. At the end of the incubation period well contents were removed and transferred to eppendorf tubes and centrifuged to pellet the cells at 400 x g for 5 minutes at 20°C. The supernatants were collected in clean tubes and stored at -20°C until analysis.

3.6.4.2 Measurement of cytokine/chemokines production by membrane array spot

A preliminary screening using a commercial kit ("*Human Cytokine Array Panel A*" R&D systems) was performed in order to evaluate which types of cytokines were produced in PBMCs after treatment with the oxysterols mixture 20 μM for 24 h.
Carefully selected capture antibodies were spotted in duplicate on nitrocellulose membranes. Cell culture supernatants (400 μl) of two samples, control and treated with the oxysterols mixture 20 μM, were diluted and mixed with a cocktail of biotinylated detection antibodies. The sample/antibody mixture was then incubated with the Human Cytokine Array Panel A membrane. Any cytokine/detection antibody complex present was bound by its analogous immobilized capture antibody on the membrane. Following a wash to remove unbound material, Streptavidin-HRP and chemiluminescent detection reagents were added sequentially. The membranes were then developed using ImageQuant

LAS 4000 mini. The light was produced at each spot in proportion to the amount of cytokine bound.

3.6.4.3 Evaluation of cytokines/chemokines (MCP-1, IL-1β, MIF and RANTES) by ELISA

After 24 h treatment, the cell culture medium was collected and centrifuged at 400 x g for 5 minutes and the supernatant stored for ELISA detection.

Levels of human cytokines MCP-1, IL-1β, MIF and RANTES were quantified using appropriate kits ("*Human CCL2 (MCP-1) ELISA Ready-SET-Go!"* eBioscience, *"Human IL-1β/IL-1F2 DuoSet*", "*Human MIF DuoSet*" and "*Human CCL5/RANTES DuoSet"* R&D systems), following the manufacturer's instructions. This assay was based on the quantitative sandwich immunoassay technique. Monoclonal antibodies, specific for the targeted cytokine, are immobilized on the internal surface of the wells of a microplate. Samples were added into the wells and the cytokine present in the sample was bound to its specific immobilized antibody. After removing any unbound compounds, an enzyme-linked polyclonal antibody specific for the cytokine was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells; the color developed in proportion to the amount of cytokines bound to its specific antibody. The intensity of the color, measured by absorbance in a microplate reader (450 nm), allowed to quantify the amount of cytokine.

3.6.5 Measurement of intracellular ROS production

PBMCs ($1x10^6$ cells/ml) were seeded in 24 well plate in complete RPMI. In the first set of experiments cells were incubated for 30 min with DCFH-DA 10 μM in the dark at 37°C. Then DCFH-DA was removed, cells were washed and incubated with the oxysterols mixture 20 μM added in fresh medium. DCFH-DA loaded cells were immediately placed in a plate reader with temperature maintained at 37°C and the fluorescence of the cells from each well was measured and recorded every 30 min until 3 h. In the second set of experiments cells were pre-treated with pure phenolic compounds, hydroxytyrosol, tyrosol and homovanillic alcohol (0.25, 0.5, 1 μM) and incubated for 30 min at 37°C in a 5% CO_2 atmosphere. Then cells were washed, treated with DCFH-DA 10 μM and incubated for 30 min in the dark at 37°C. After DCFH-DA was removed, cells were washed and incubated with the oxysterols mixture 20 μM added in fresh medium for 2 h. The fluorescence of the cells from each well was then measured, setting the excitation filter at 485 nm and the emission filter at 530 nm.

3.6.6 Modulation of intracellular signaling pathways

3.6.6.1 Cells treatment

PBMCs ($5x10^6$ cells/ml) were seeded in 6 well plate in complete RPMI, pre-treated with pure phenolic compounds, hydroxytyrosol, tyrosol and homovanillic alcohol (0.25, 0.5, 1 μM) and incubated for 30 min at 37°C in a 5% CO_2 atmosphere. The oxysterols mixture 20 μM was then added in the medium and cells incubated for 3 h.

3.6.6.2 Extraction and quantification of proteins

At the end of the incubation time well contents were collected in appropriate centrifuge tubes and centrifuged at 400 x g for 5 min at 4°C. Supernatants were discarded and cells washed/centrifuged twice with cold PBS. Finally pellets were suspended with 150 μL of complete lysis buffer (with protease inhibitors) and incubated on ice for 30 min (by vortexing every 10 min). At the end, lysates were centrifuged at 15000 x g for 5 min at 4°C to remove any particles or cell debris and the supernatants were collected for protein analysis.
The protein concentration was determined by the Bradford protein assay **(Bradford 1976)**.

3.6.6.3 Electrophoresis and immunoblotting

The procedure was conducted as previously described (section 3.5.8.3).

The blots were incubated with these specific antibodies: anti-pJNK1/2 (1:200 dilution), anti-JNK 1/2 (1:200 dilution), anti-pp38 (1:1000 dilution) and anti-p38 (1:1000 dilution) in TTBS containing 1% (w/v) skimmed milk powder (antibody buffer).

3.7 Statistical analysis

Results are expressed as means ± S.D. (standard deviation). The statistical significance of parametric differences among sets of experimental data was evaluated by the one-way ANOVA test associated with Bonferroni's multiple comparison post test using GraphPad InStat (GraphPad Software, San Diego, CA, USA).

4. Results

4.1 Pro-oxidant and pro-inflammatory activity of oxysterols in the gut and protective effect of EVOO phenolic extracts

4.1.1 Intracellular ROS production

ROS generation in Caco-2 cells was determined using the fluorescent probe DCFH-DA, which can be oxidised to the highly fluorescent compound DCF in the presence of ROS.

Before treatments, cells were incubated with 10 μM of DCFH-DA for 30 min at 37°C in the dark, then were treated using different concentrations of oxysterols mixture (60-90-150 μM) for different incubation times (0-180 min).

The incubation with the oxysterols mixture, as shown in Figure 7, induced a significant increase of intracellular ROS production from 90 μM concentration after 60 minutes of incubation.

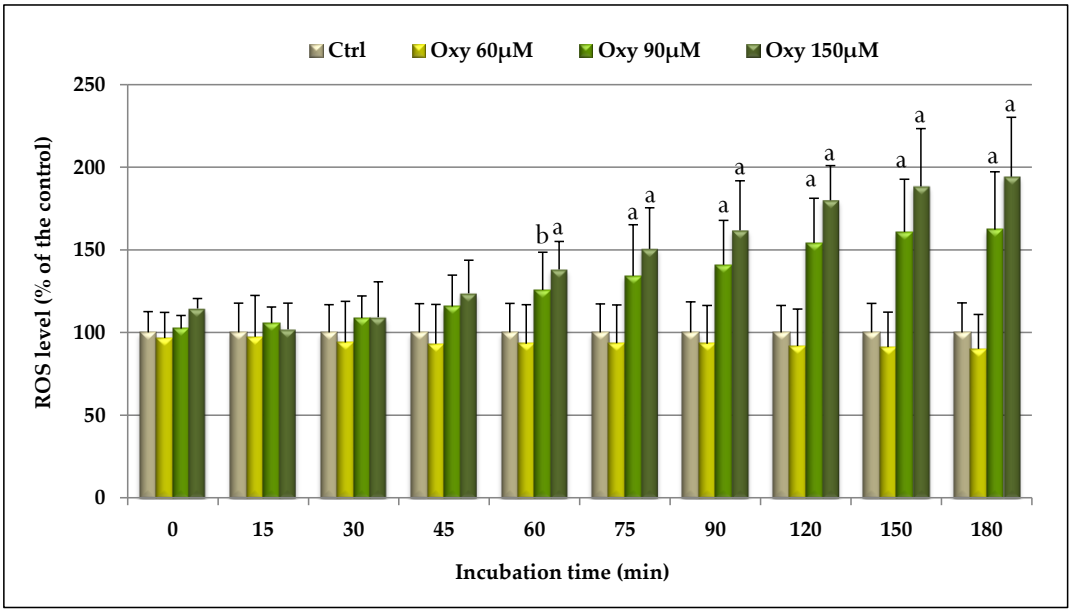


Figure 7: Intracellular ROS production (expressed as % of control) in Caco-2 cells treated with different concentrations of the oxysterols mixture (60-90-150 μM) for different incubation times (0-180 min) using the fluorescence probe DFC-DA 10 μM for 30 minutes.
a =p<0.001, b =p<0.01 vs Ctrl (n=12)

The presence of the phenolic extracts (5-10-25 μM), added simultaneously with the oxysterols mixture 150 μM for 1h, induced a significant decrease of ROS production all tested concentrations, as shown in Figure 8.

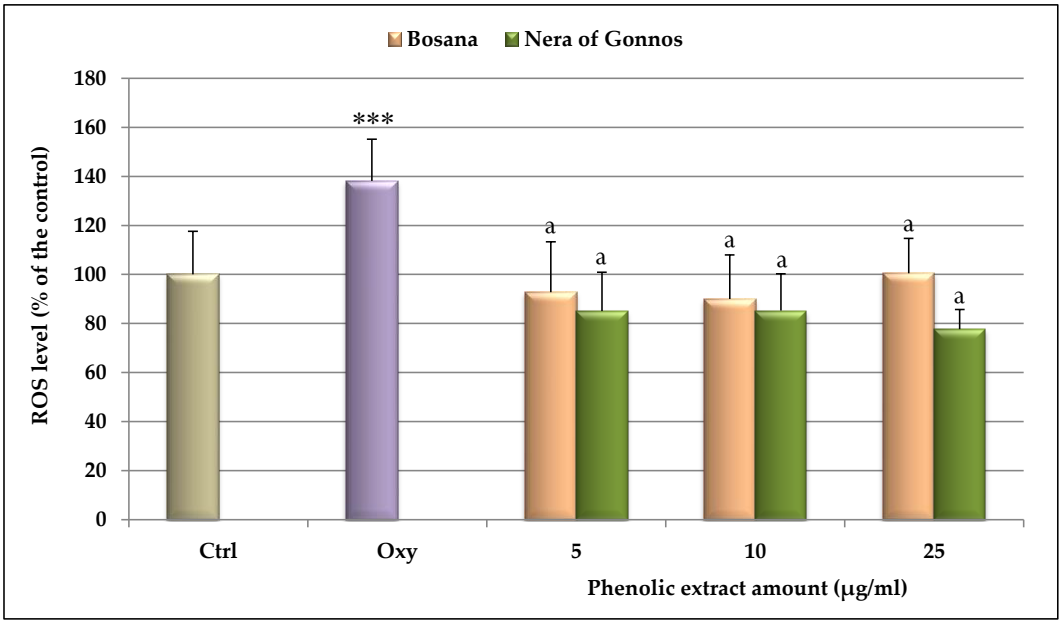


Figure 8: Effects of Bosana and Nera of Gonnos phenolic extracts (5-10-25 μg/ml) on intracellular ROS production in Caco-2 cells treated with the oxysterols mixture 150 μM for 1 h.
*** = $p<0.001$ vs Ctrl; a = $p<0.001$ vs Oxy (n=12)

4.1.2 Quantification of glutathione (GSH)

To evaluate the redox state in Caco-2 cells, after ROS production, the level of cellular GSH was quantified. As reported in Figure 9 cells were incubated with the oxysterols mixture 150 μM for different times (30 min-24h); the presence of the mixture resulted in a significant decrease of the concentration of GSH after 30 minutes of incubation. After 3 h the concentration increased, returning to control values after 6 hours.

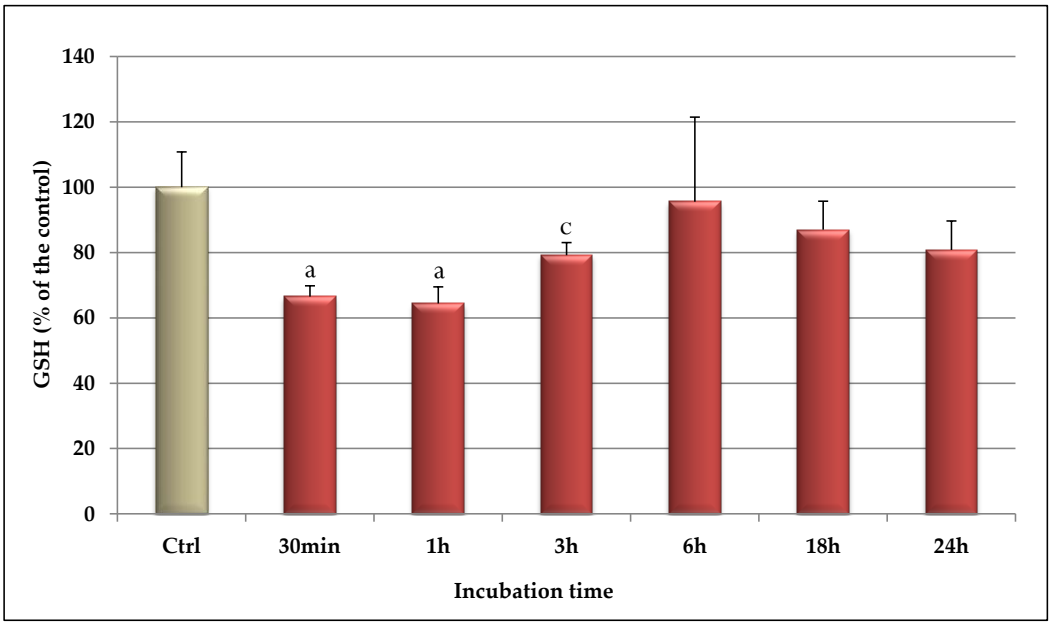


Figure 9: GSH concentration, expressed as % of the control, in Caco-2 cells incubated for different times (30 min-24h) with the oxysterols mixture 150 μM.
a = $p<0.001$, c = $p<0.05$ vs Ctrl (n=9) Control value = 55.4 μM

Figure 10 shows GHS levels (expressed as % of control) in Caco-2 cells pre-treated with the phenolic extracts (5-10-25 μg/ml) for 30 min, and treated with the oxysterols mixture 150 μM for other 30 min.

The decrease of GSH induced by the oxysterols mixture was significantly counteracted by both phenolic extracts from the concentration of 10 μg/ml.

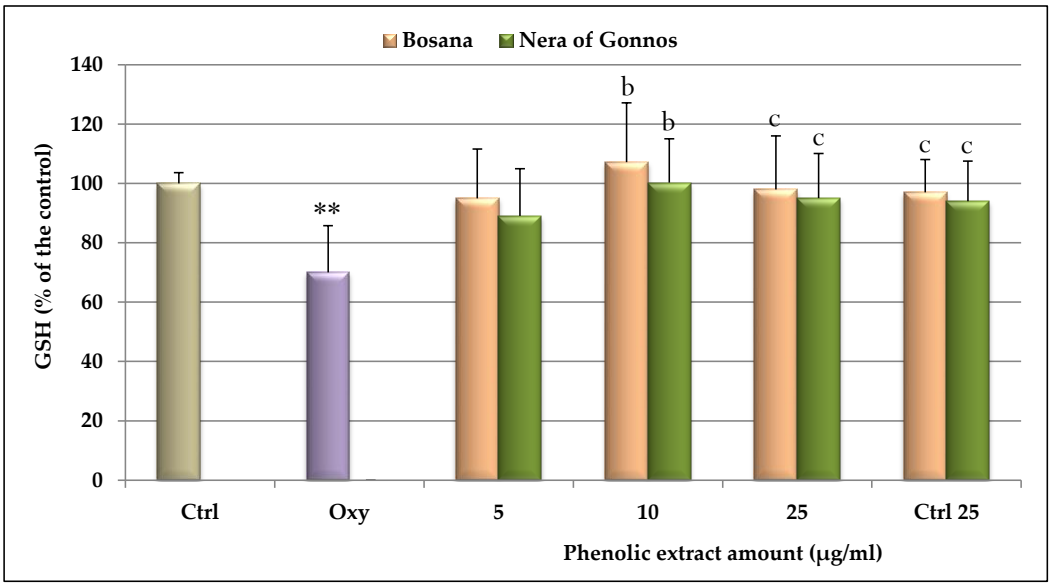


Figure 10: GSH concentration (expresses as % of control) in Caco-2 cells pre-treated 30 min with Bosana and Nera of Gonnos phenolic extracts (5-10-25 µg/ml) and treated with the oxysterols mixture 150 µM for 30 min.
**=p<0.01 vs Ctrl; b= p<0.01, c=p<0.05 vs Oxy (n=9) Control value = 28 µM

4.1.3 Modulation of glutathione peroxidase (GPX) activity

The modulatory effect of the oxysterols mixture on the activity of GPX in Caco-2 cells was also determined.

Figure 11 shows the GPX activity, expressed as % of the control, in Caco-2 cells incubated for different times (6-36 hours) with the oxysterols mixture 150 μM. The mixture induced a significant increase of the enzyme activity after 6, 18 and 24 hours, but after 36 hours of incubation the enzyme activity decreased to the control value.

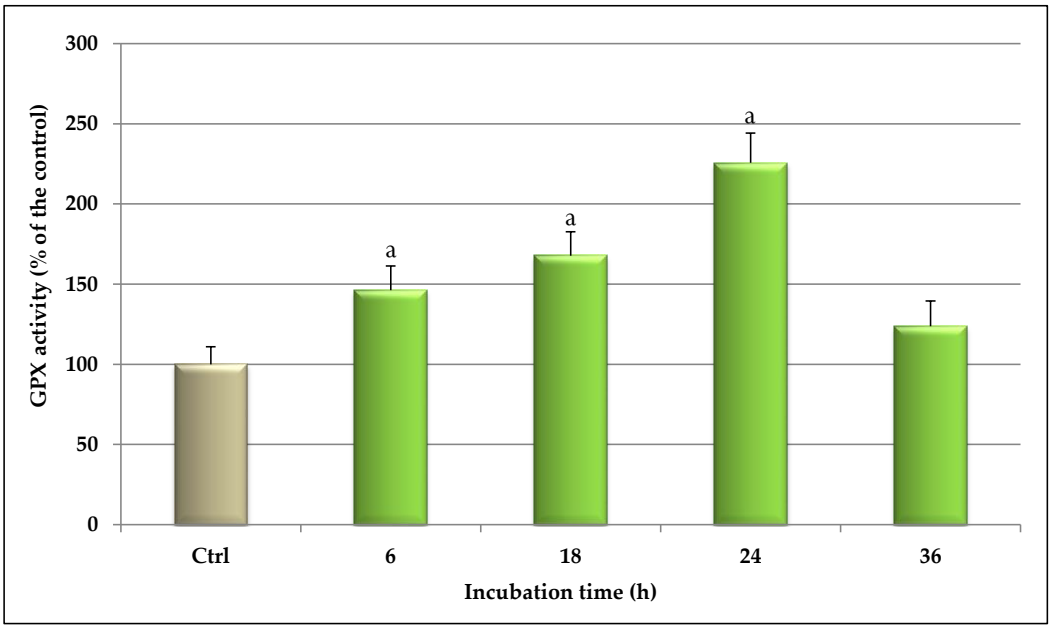


Figure 11: Glutathione peroxidase activity (GPX), expressed as % of the control, in Caco-2 cells incubated for different times (6-36 min) with the oxysterols mixture 150 μM.
a = $p<0.001$ vs Ctrl (n=9) Control value = 44.4 mmol/min/ml

When Caco-2 cells were incubated with the phenolic extracts (5-10-25 μg/ml) for 30 min, before the treatment with the oxysterols mixture 150 μM for 18 h, a significant decrease of the GPX activity was observed from 10 μg/ml for both extracts (Figure 12).

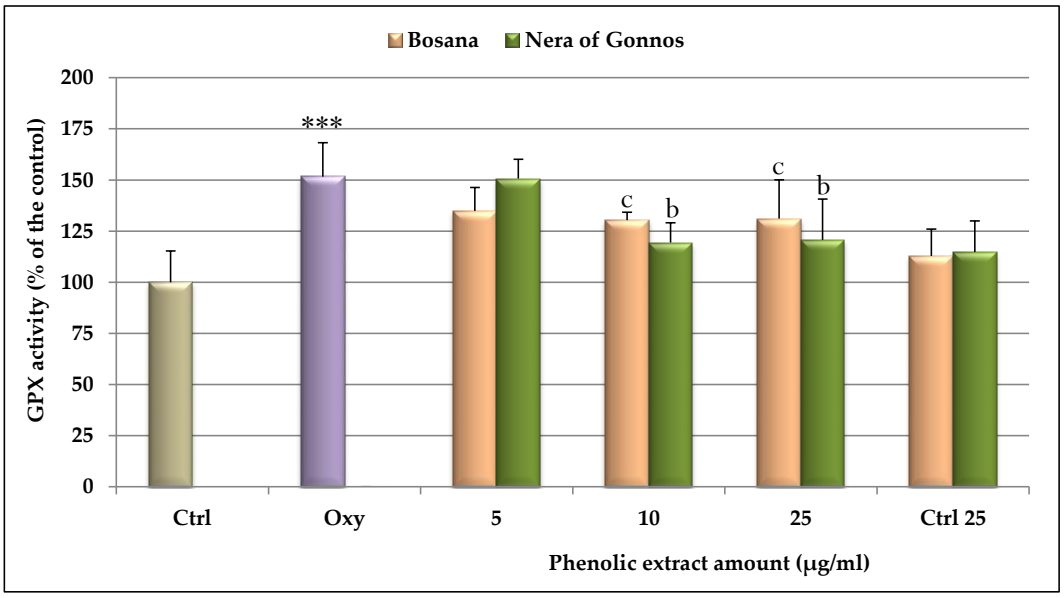


Figure 12: Glutathione peroxidase activity (GPX), expressed as % of the control, in Caco-2 cells pre-treated with the phenolic extracts (5-10-25 µg/ml) for 30 min and treated for 18 h with the oxysterols mixture 150 µM.
*** = $p<0.001$ vs Ctrl; b = $p<0.01$, c = $p<0.05$ vs Oxy (n=9) Control value = 27 mmol/min/ml

4.1.4 Measurement of malondialdehyde (MDA) production

In order to evaluate any oxidative damage induced by oxysterols in Caco-2 cells the amount of malondialdehyde produced was quantified.

MDA is a secondary product of membrane lipids peroxidation and is considered a generic marker of oxidative damage. MDA levels in supernatants of treated cells were measured by the TBARS test with HPLC quantification.

Figure 13 shows the amount of MDA, expressed as percentage compared to control, determined in cells treated for 24 hours with different concentrations of the mixture of oxysterols (30-150 μM). As shown, oxysterols induced an increase of MDA production compared to control significant from the concentration of 90 μM.

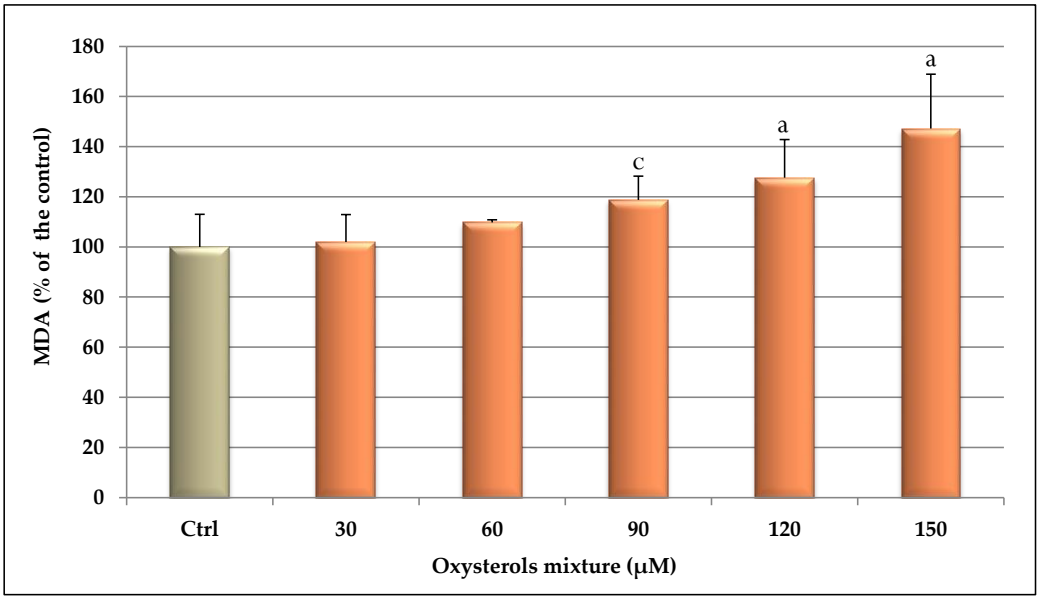


Figure 13: Percentage of MDA production compared to control in Caco-2 cells incubated for 24 h with different concentrations (30-150 μM) of the oxysterols mixture.
a = $p<0.001$, c = $p<0.5$ vs Ctrl (n=12)

The presence of the phenolic extracts, was able to significantly reduce, at all tested concentrations (5-10-25 μg/ml), the amount of MDA produced in Caco-2 cells after 24 h incubation with oxysterols 150 μM, as shown in Figure 14.

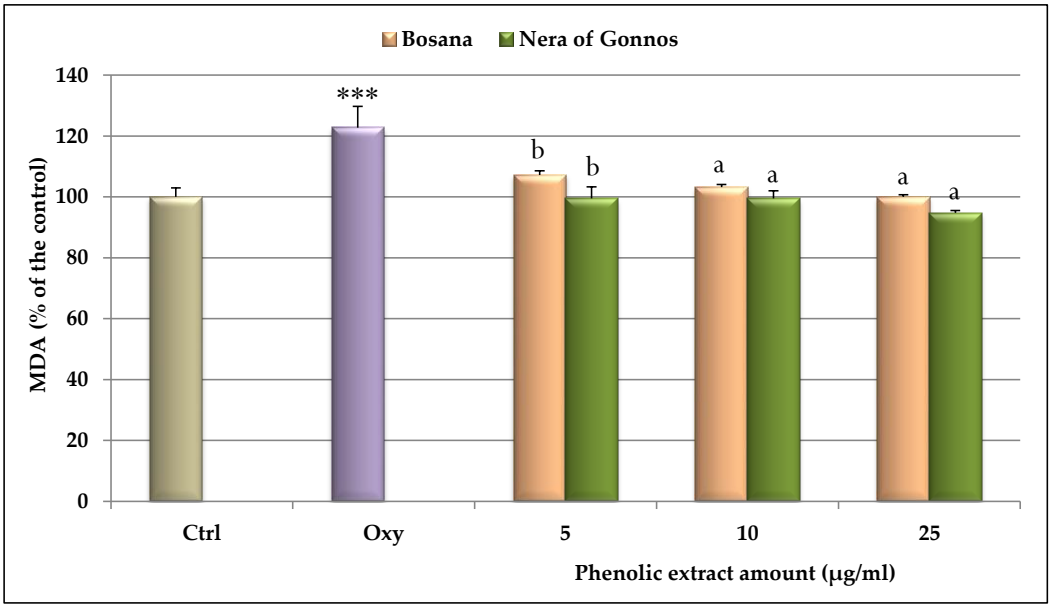


Figure 14: Percentage of MDA production compared to control in Caco-2 cells pre-treated for 30 min with different amounts of the phenolic extracts (5-10-25 μg/ml) and incubated for 24 h with the oxysterols mixture 150 μM.
*** = $p<0.001$ vs Ctrl; a = $p<0.001$, b = $p<0.01$ vs Oxy (n=12)

4.1.5 Cell viability

The protective activity of EVOO phenolic extracts was also evaluated against the cytotoxic action of the oxysterols mixture in Caco-2 cells. Cell viability was measured by the Neutral Red test and expressed as percentage of viability compared to the control (100% viability).

Figure 15 shows the percentage of Caco-2 cells viability incubated for 24 hours with increasing concentrations of the oxysterols mixture (30-150 μM). The presence of the mixture induced a decrease of viability compared to control, that was significant from 60 μM.

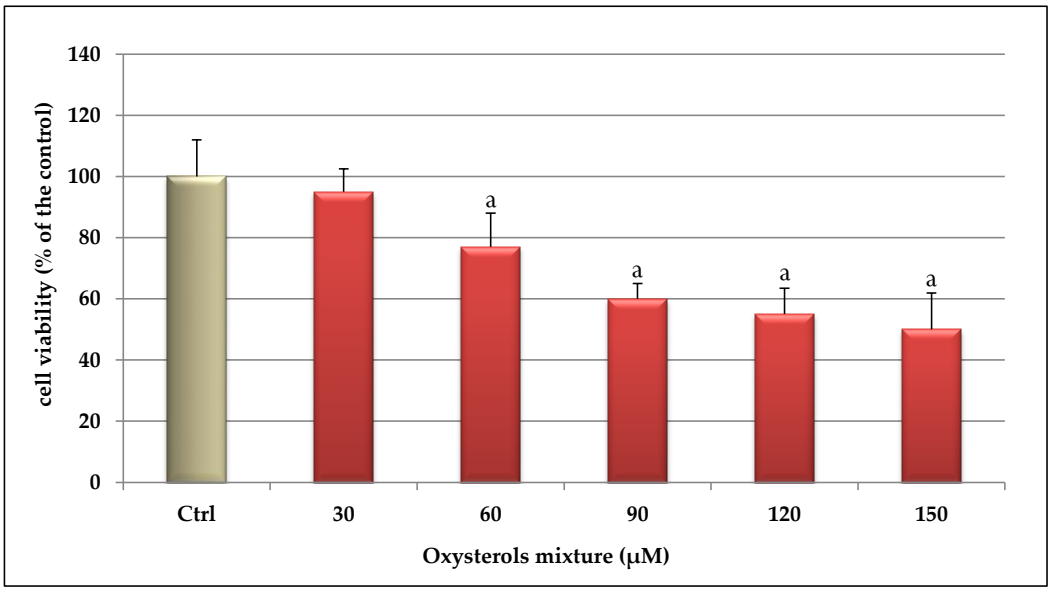


Figure 15: Percentage of cell viability compared to control of Caco-2 cell incubated for 24 h with different concentrations of the oxysterols mixture (30-150 μM).
a = p<0.001 vs Ctrl (n=9)

In order to evaluate the protective activity of EVOO phenolic extracts, cells were pre-treated for 30 min with the phenolic extracts of Bosana and Nera of Gonnos (1-5-10-25 μg/ml) and treated with the oxysterols mixture 150 μM for 24 h.

Both phenolic extracts were able to significantly inhibit the citotoxicity of oxysterols at all tested concentrations, as shown in Figure 16.

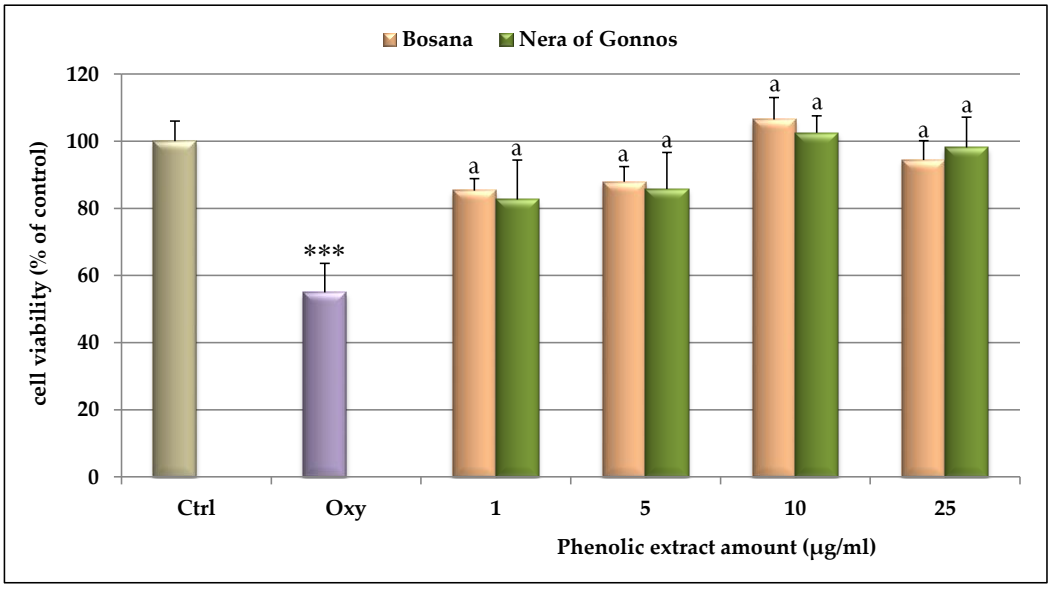


Figure 16: Percentage of viability compared to control of Caco-2 cells pre-treated with Bosana and Nera of Gonnos phenolic extracts (1-25 µg/ml) and incubated for 24 h with the oxysterols mixture 150 µM.
*** = $p<0.001$ vs Ctrl; a = $p<0.001$ vs Oxy (n=9)

4.1.6 Modulation of intracellular signaling pathways

The oxysterols mixture was able to induce cytotoxicity in Caco-2 cells, reducing cell viability significantly from 60 μM concentration; the mechanism involved in cell death was then analysed.

The ability of the oxysterols to interfere with intracellular signaling pathways involved in programmed cell death (apoptosis) was evaluated, in particular focusing on caspase 3, Akt/PKB, ERK 1/2, JNK 1/2 and p38 modulation.

4.1.6.1 Caspase 3

Figure 17 shows the caspase 3 activity in Caco-2 cells incubated for 24 h with different concentrations of the oxysterols mixture (30-150 μM). The mixture was able to induce a significant increase of caspase 3 activity from the concentration of 60 μM.

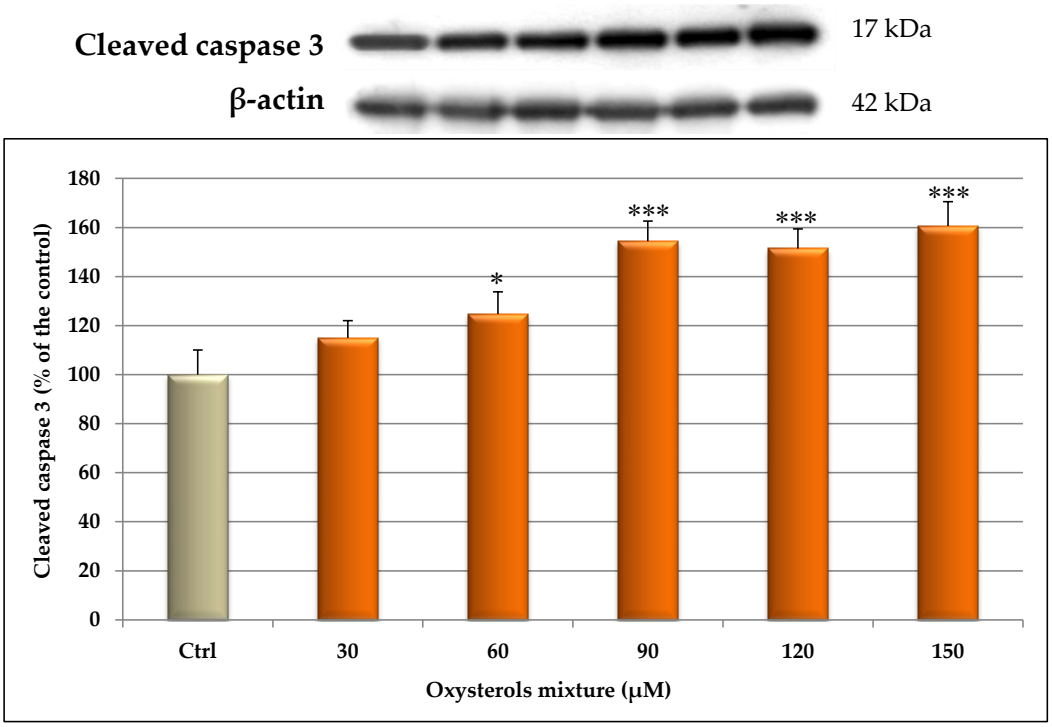
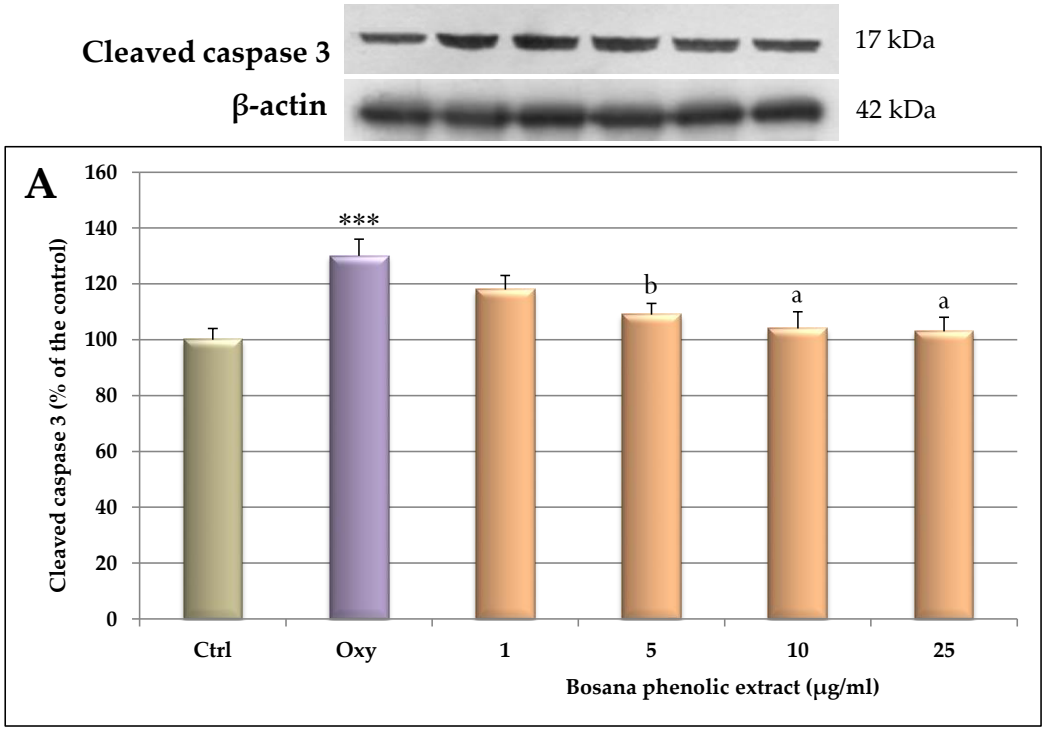


Figure 17: Caspase 3 activity in Caco-2 cells after 24 h incubation with different concentrations of the oxysterols mixture (30-150 μM).
***= p<0.001,* = p<0.05 vs Ctrl (n=3)

To evaluate the capability of phenolic extracts to counteract the oxysterols induced caspase 3 increased activity, cells were pre-treated for 30 min with the phenolic extracts (1-5-10-25 μg/ml) and treated with the oxysterols mixture 60 μM for 24 h. Both phenolic extracts were able to protect intestinal cells from oxysterols induced death, in particular, the Bosana phenolic extract significantly from the concentration of 5 μg/ml (Figure 18 A) and the Nera of Gonnos phenolic extract from 10 μg/ml (Figure 18 B).



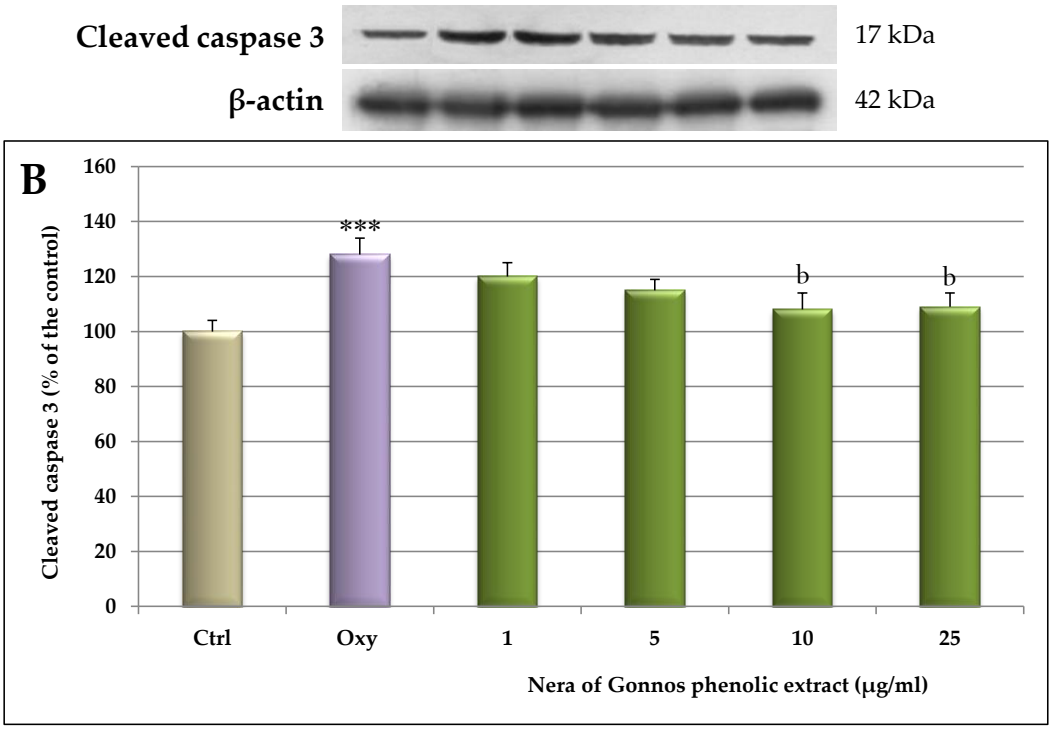


Figure 18: Caspase 3 activity in Caco-2 cells pre-treated with Bosana (A) and Nera of Gonnos (B) phenolic extracts (1-25 μg/ml) and treated for 24 h with the oxysterols mixture 150 μM.
*** = $p < 0.001$ vs Ctrl; a = $p<0.001$, b = $p<0.01$ vs Oxy (n=3)

4.1.6.2 Akt/PKB

Figure 19 shows the change of Akt/PKB phosphorilation state in Caco-2 cells treated with different concentrations of the oxysterols mixture (30-150 μM) for 30 min. The band values were normalized using the corresponding values of total protein and expressed as a percentage of the control. Oxysterols induced a significant decrease of Akt/PKB phosphorilation compared to control from 60 μM.

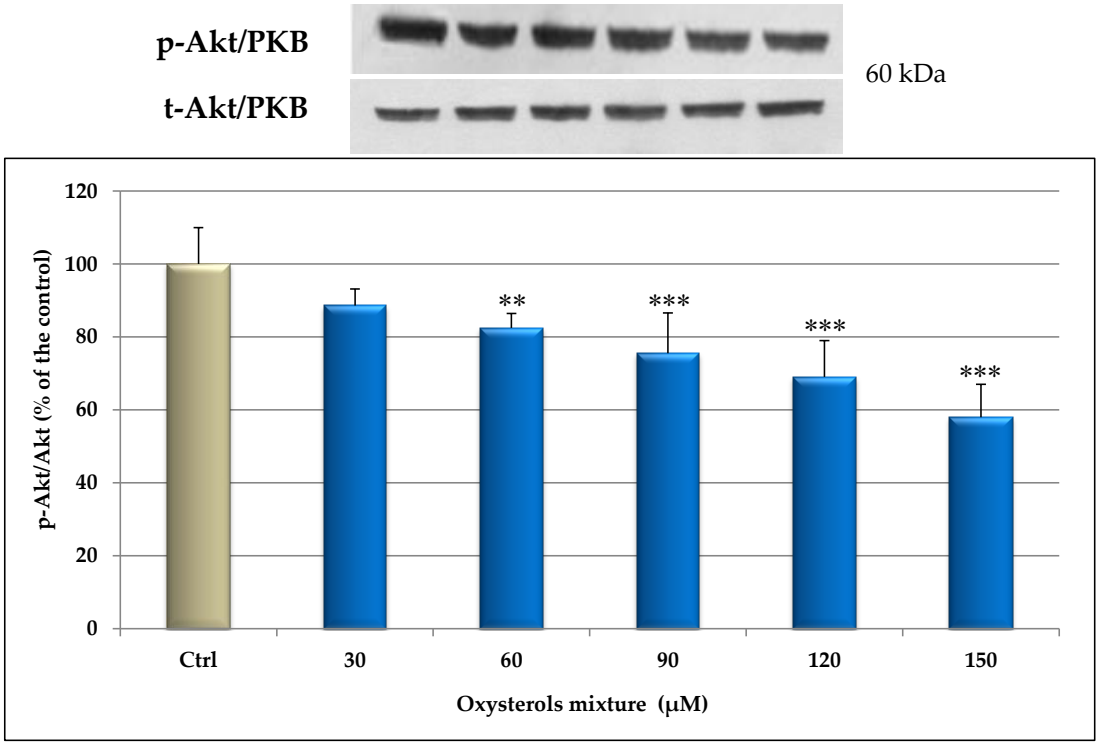


Figure 19: Modulation of Akt/PKB in Caco-2 cell monolayers after 30 min incubation with different concentrations of the oxysterols mixture (30-150 μM).
***= p<0.001, ** = p<0.01vs Ctrl (n=3)

Pre-treatment with both phenolic extracts (1-5-10-25 μg/ml) significantly inhibited the decrease of Akt/PKB phosforilation induced by the oxysterols, from the lowest tested concentration (Figure 20 A, B).

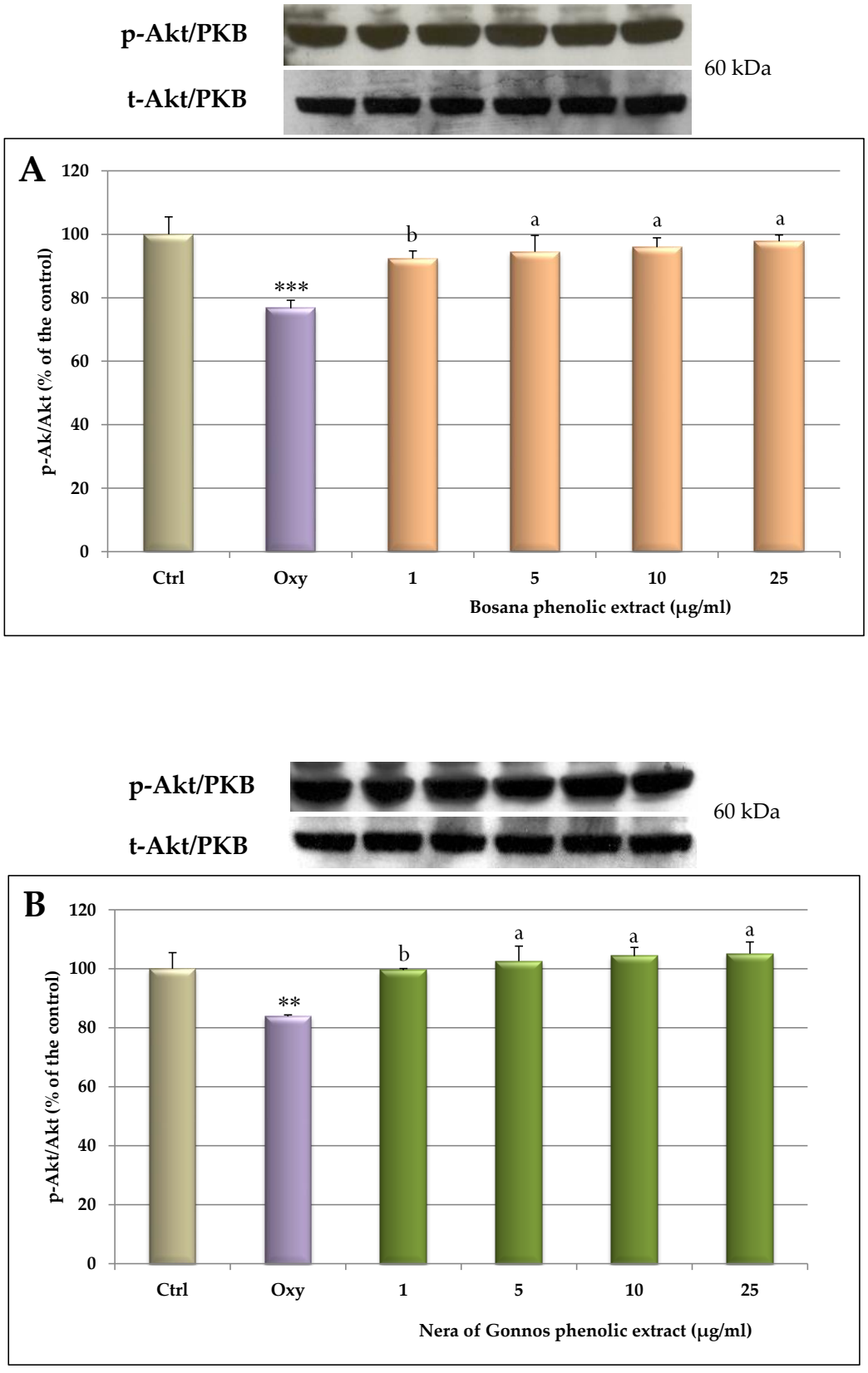


Figure 20: Modulation of Akt/PKB in Caco-2 cell monolayers pre-treated with Bosana (A) and Nera of Gonnos (B) phenolic extracts for 30 min and treated with the oxysterols mixture 60 µM.
*** = $p<0.001$, ** = $p< 0.01$ vs Ctrl; a = $p<0.001$, b = $p<0.01$ vs Oxy (n=3)

4.1.6.3 ERK 1/2

Figure 21 shows the modulation of ERK 1/2 phosphorilation in Caco-2 cells treated with different concentrations of the oxysterols mixture (30-150 μM) for 30 min. The band values were normalized using the corresponding values of total protein and expressed as a percentage of the control. Oxysterols induced a significant reduction of ERK 1/2 phosphorilation compared to control, from the 60 μM.

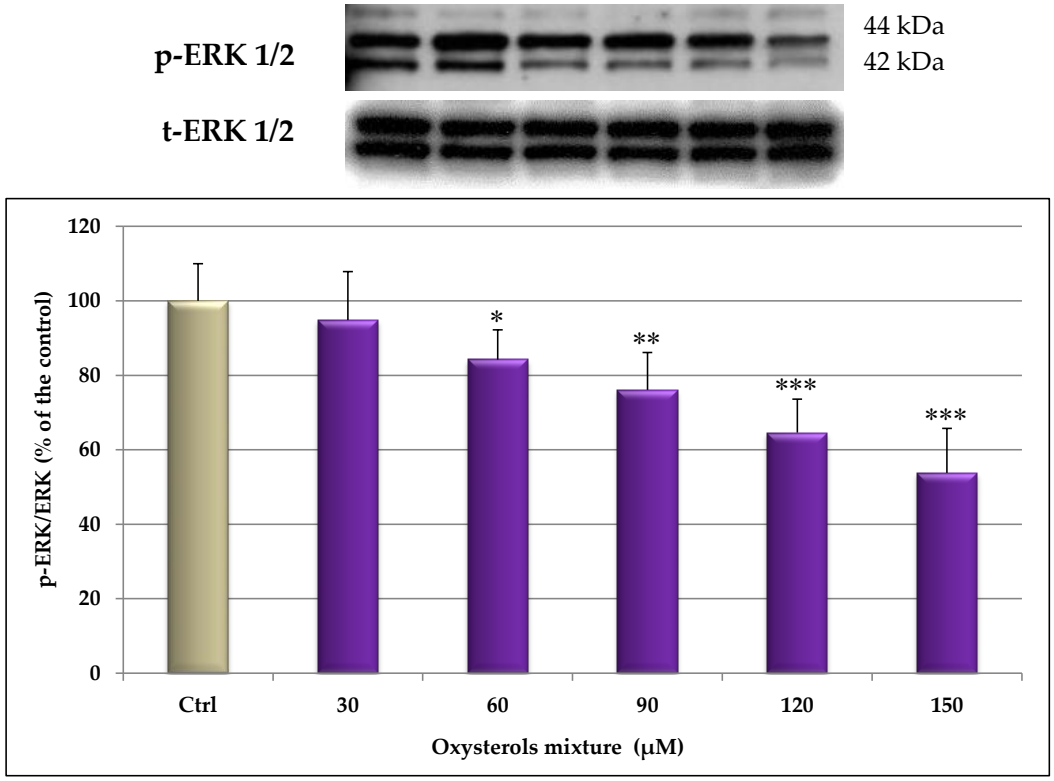


Figure 21: Modulation of ERK 1/2 in Caco-2 cell monolayers after 30 min of incubation with different concentrations of the oxysterols mixture (30-150 μM).
***= $p<0.001$, ** = $p<0.01$, * = $p<0.05$ vs Ctrl (n=5)

Pre-treatment with both phenolic extracts (1-5-10-25 μg/ml) significantly inhibited the decrease of ERK 1/2 phosphorilation induced by the oxysterols (Figure 22 A, B).

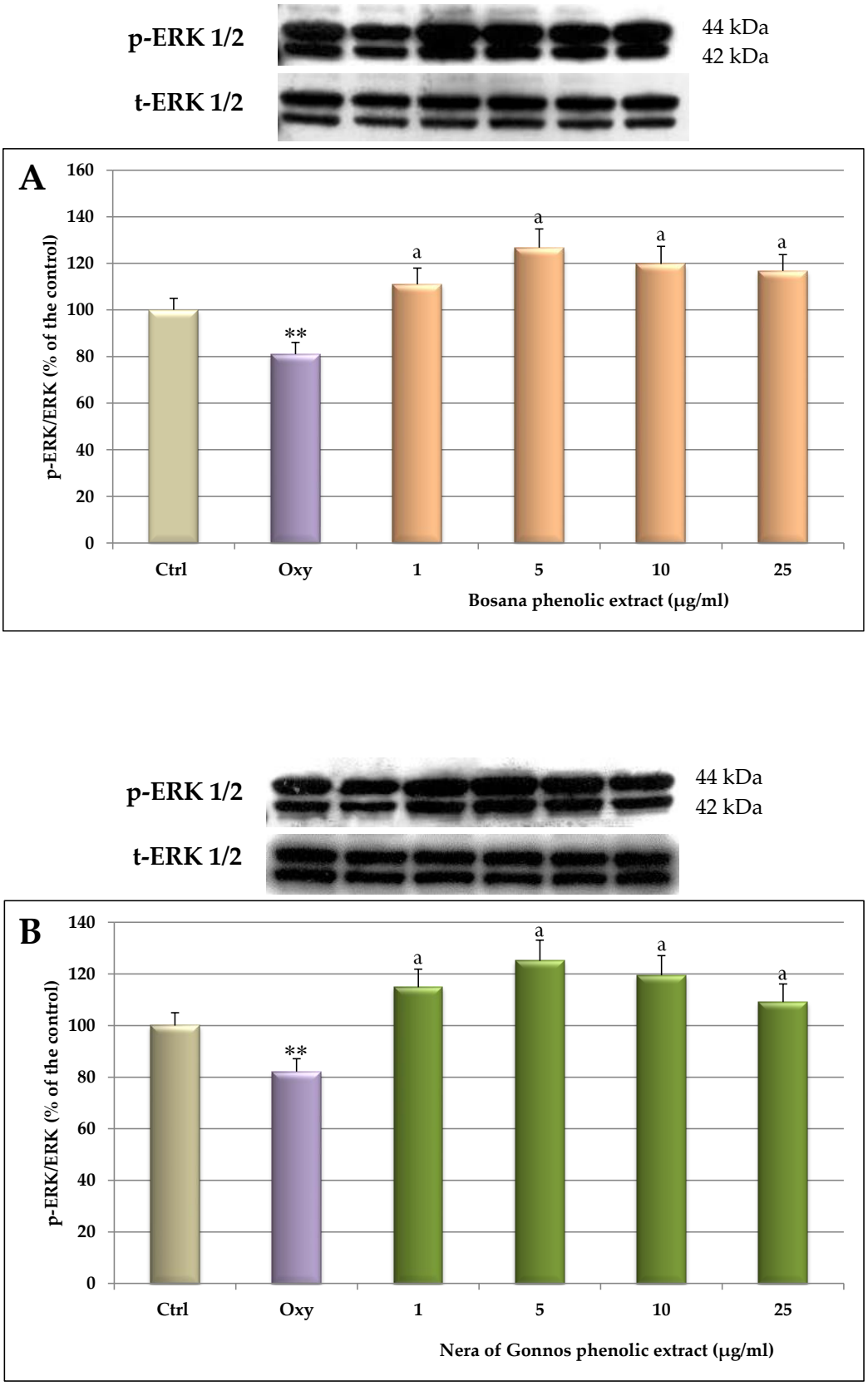
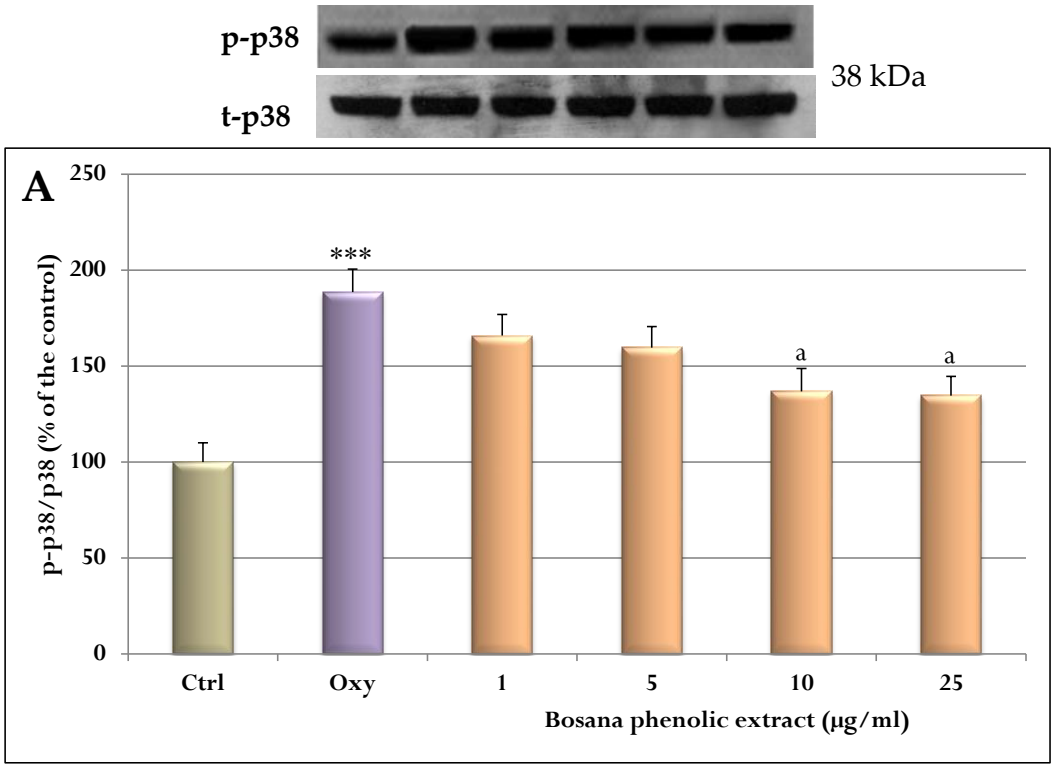


Figure 22: Modulation of Erk 1/2 in Caco-2 cell monolayers pre-treated with Bosana (A) and Nera of Gonnos (B) phenolic extracts for 30 min and treated with the oxysterols mixture 60 µM.
**= $p<0.01$ vs Ctrl; a = $p<0.001$ vs Oxy (n=3)

4.1.6.4 P38

Figure 23 shows the modulation of p38 phosphorilation in Caco-2 cells pre-treated with different concentrations of the phenolic extract (1-5-10-25 μg/ml) for 30 min and treated with the oxysterols mixture 60 μM for 30 min. The band values were normalized using the corresponding values of total protein and expressed as a percentage of the control. Oxysterols induced a significant increase of p38 phosphorilation compared to control, that was counteracted by both Bosana and Nera of Gonnos phenolic extracts at maximum tested concentration. Bosana extract reduced this increase significantly also at the concentration of 10 μg/ml (Figure 23 A, B).



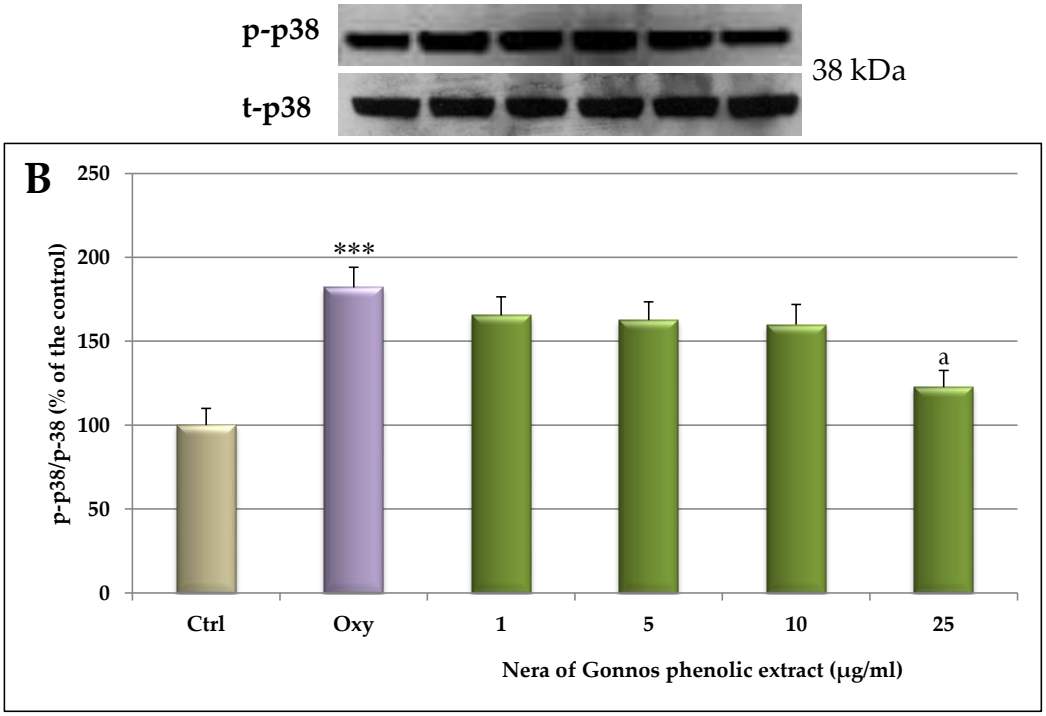
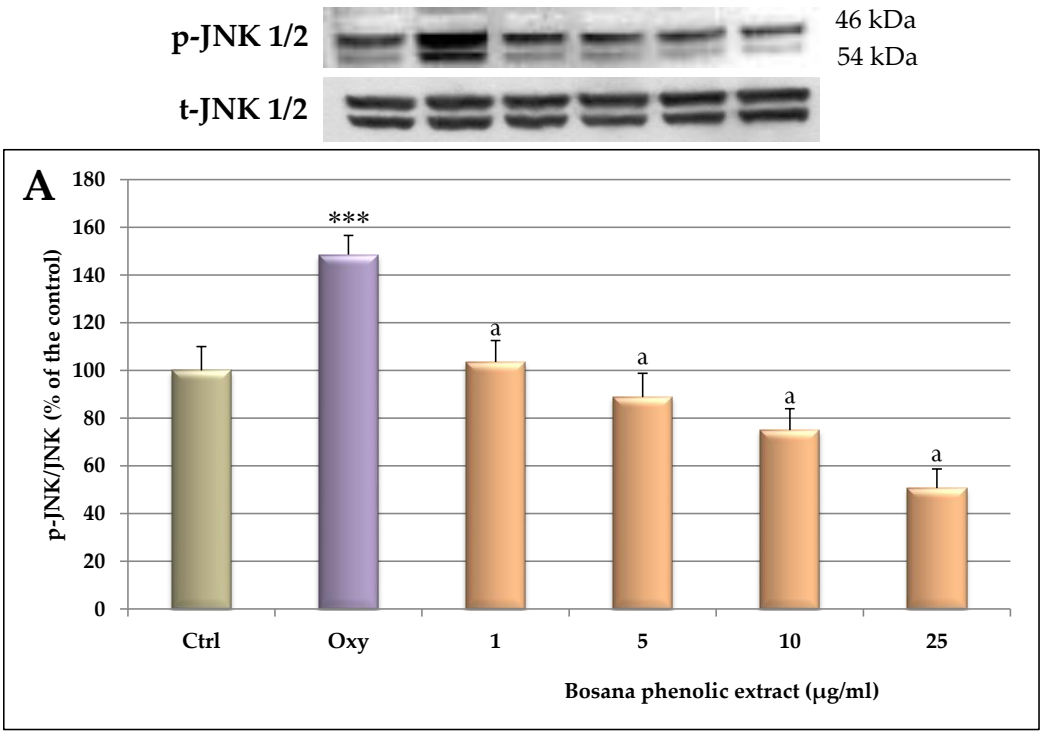


Figure 23: Modulation of p38 in Caco-2 cell monolayers cells pre-treated or not for 30 min with Bosana (A) and Nera of Gonnos (B) phenolic extracts (1-5-10-25 µg/ml) and incubated with the oxysterols mixture 60 µM for 30 min.
***= $p<0.001$ vs Ctrl; a = $p<0.001$ vs Oxy (n=3)

4.1.6.5 JNK 1/2

Figure 24 shows the modulation of JNK 1/2 phosphorilation in Caco-2 cells pre-treated with different concentrations of the phenolic extract (1-5-10-25 μg/ml) for 30 min and treated with the oxysterols mixture 60 μM for 30 min. The band values were normalized using the corresponding values of total protein and expressed as a percentage of the control. Oxysterols induced a significant increase of JNK phosphorilation compared to control; both Bosana and Nera of Gonnos phenolic extracts significantly reduced this increase at all tested concentrations (Figure 24 A, B).



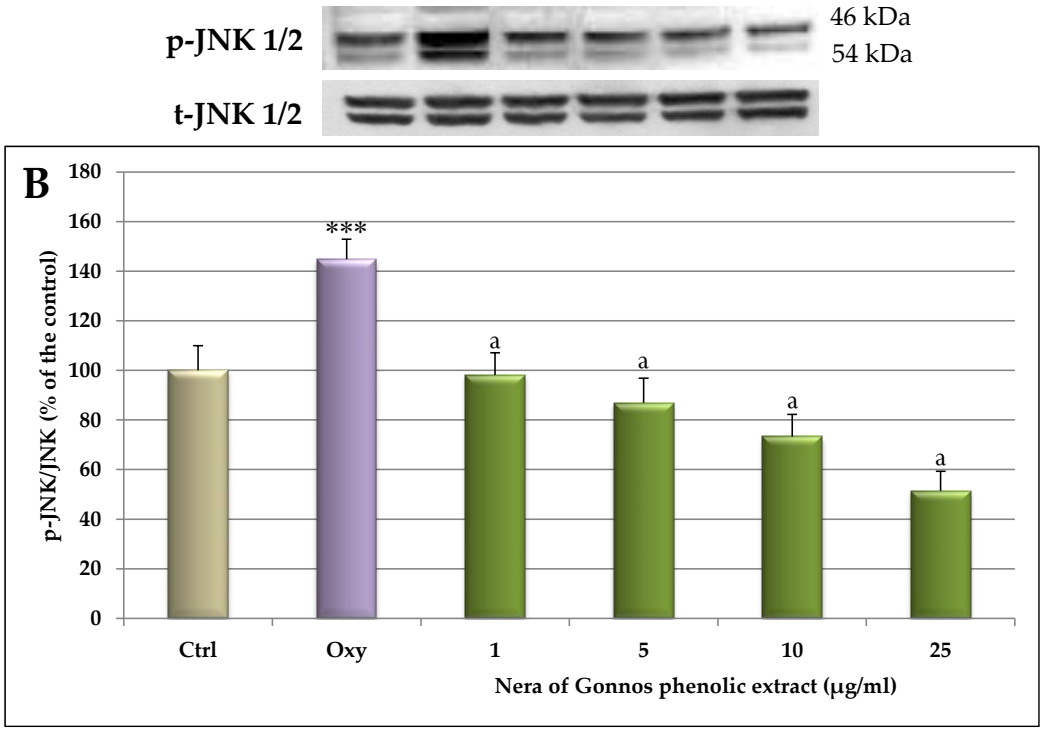


Figure 24: Modulation of JNK 1/2 in Caco-2 cell monolayers cells pre-treated or not for 30 min with Bosana (A) and Nera of Gonnos (B) phenolic extracts (1-5-10-25 µg/ml) and incubated with the oxysterols mixture 60 µM for 30 min.
***= $p<0.001$ vs Ctrl; a = $p<0.001$ vs Oxy (n=3)

4.1.7 Induction of pro-inflammatory cytokines synthesis: IL-8 and IL-6

The activation of MAPK pathway is involved not only in apoptosis process but leads to the induction of a wide range of genes that contribute to the establishment of the immune response and inflammation, regulating the synthesis/activity of cytokines and chemokines, cell surface receptors, adhesion molecules and inflammatory enzymes.

The capability of the mixture to induce the synthesis of interleukin 8 (IL-8) and interleukin 6 (IL-6) was also evaluated in Caco-2 cells together with the ability of phenolic extracts (Bosana and Nera of Gonnos 25μg/ml) to counteract this effect. Cytokine concentration, expressed as percentage compared to control, was evaluated by ELISA method in the culture medium.

Treatment of Caco-2 cells with the oxysterols mixture 60 μM for 24 h induced a significant increase of the synthesis of IL-8. Pre-treatment for 30 min with both phenolic extracts significantly reduced interleukin synthesis induced by the oxysterols (Figure 25).

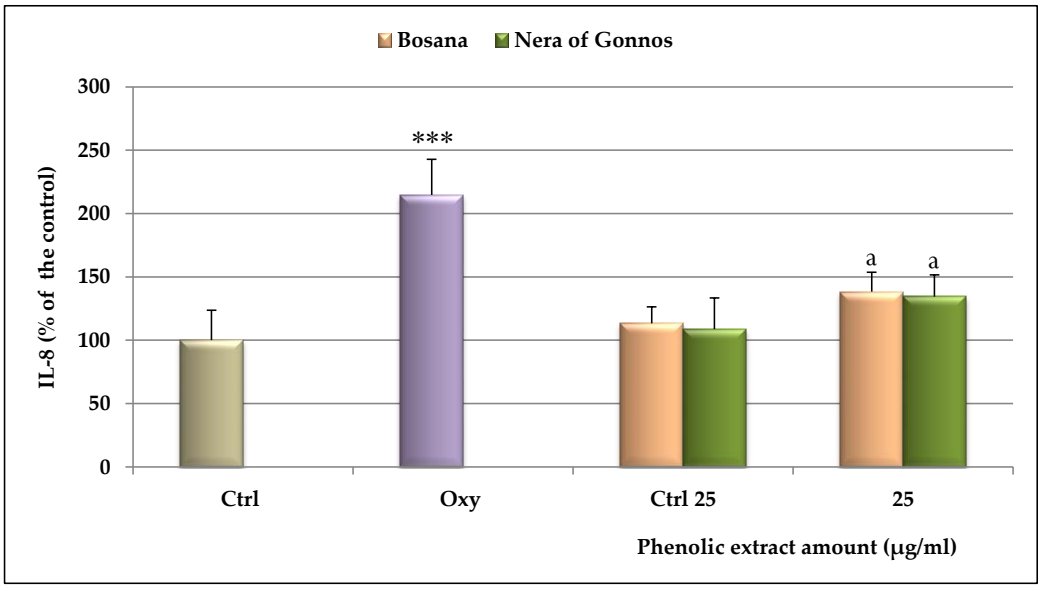


Figure 25: IL-8 synthesis, expresses as % compared to control, in Caco-2 cells pre-treated or not for 30 min with Bosana and Nera of Gonnos phenolic extracts (25 μg/ml) and incubated with the oxysterols mixture 60 μM for 24 h.
*** = p<0.001 vs Ctrl; a = p<0.001 vs Oxy (n=5) Control value = 6.23 pg/ml

The oxysterols mixture were also able to increase the production of IL-6 (Figure 26) and, as shown, Bosana and Nera of Gonnos phenolic extracts significantly reduced this effect.

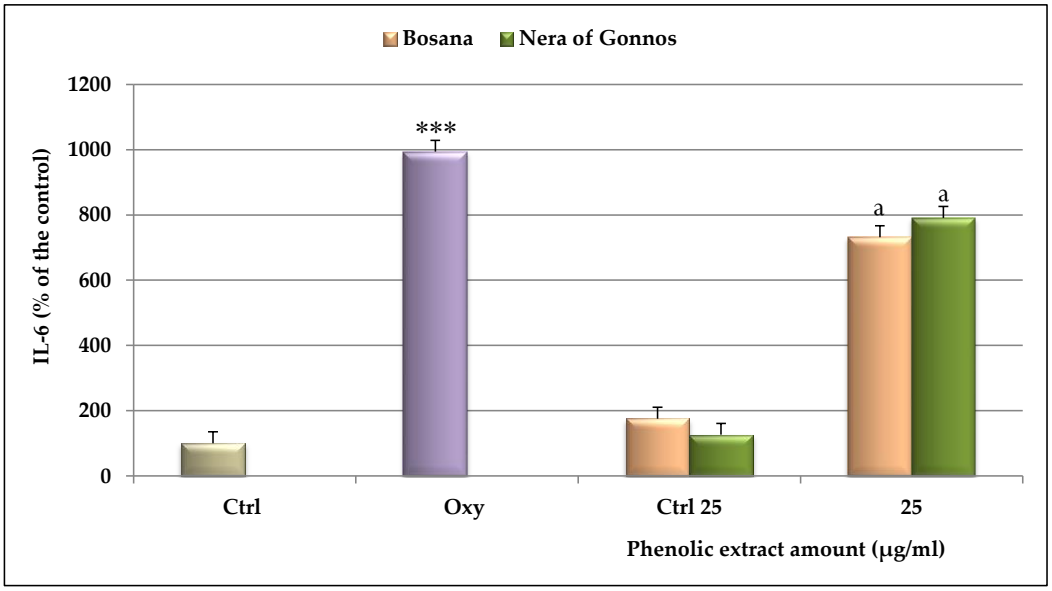


Figure 26: IL-6 synthesis, expressed as % compared to control, in Caco-2 cells pre-treated or not for 30 min with Bosana and Nera of Gonnos phenolic extracts (25 µg/ml) and incubated with the oxysterols mixture 60 µM for 24h.
*** = $p<0.001$ vs Ctrl; a = $p<0.001$ vs Oxy (n=5) Control value = 2.87 pg/ml

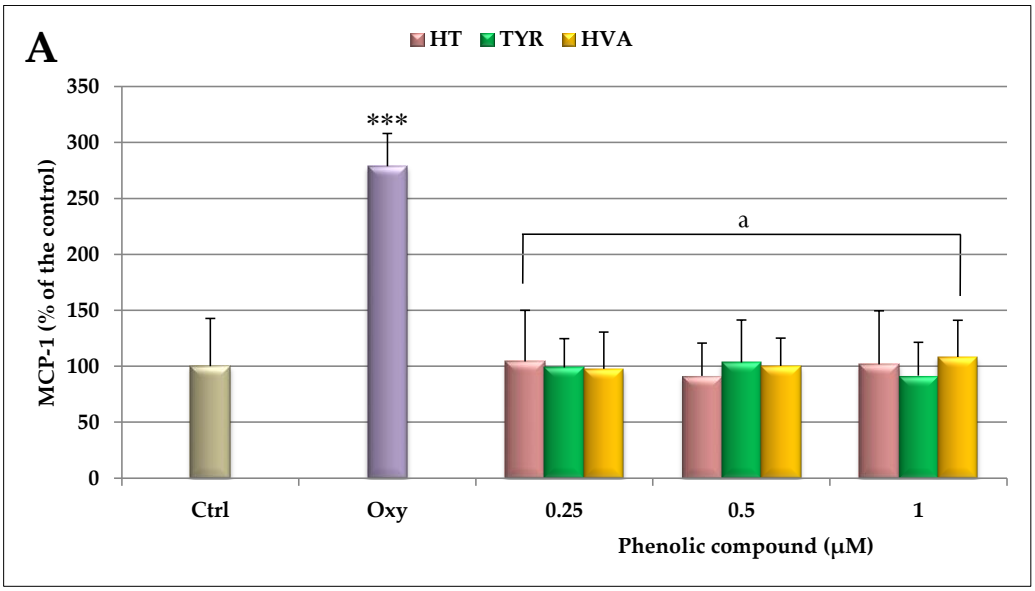
4.2 Pro-oxidant and pro-inflammatory activity of oxysterols in PBMCs and protective effects of hydroxytyrosol, tyrosol and homovanillic alcohol

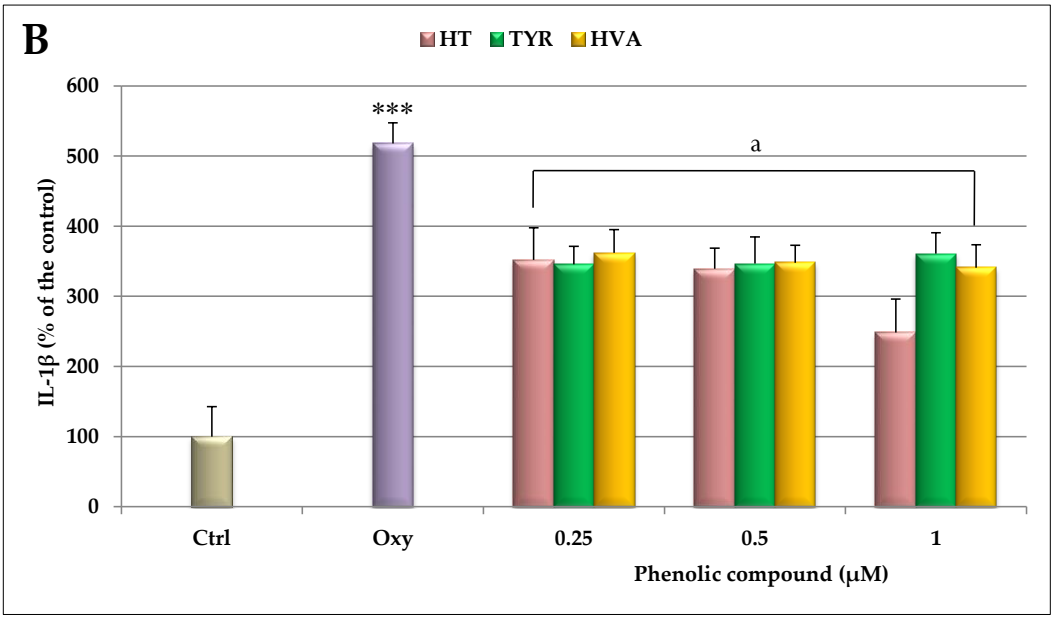
4.2.1 Induction of the synthesis of pro-inflammatory cytokines/chemokines: MCP-1, IL-1β, MIF and RANTES

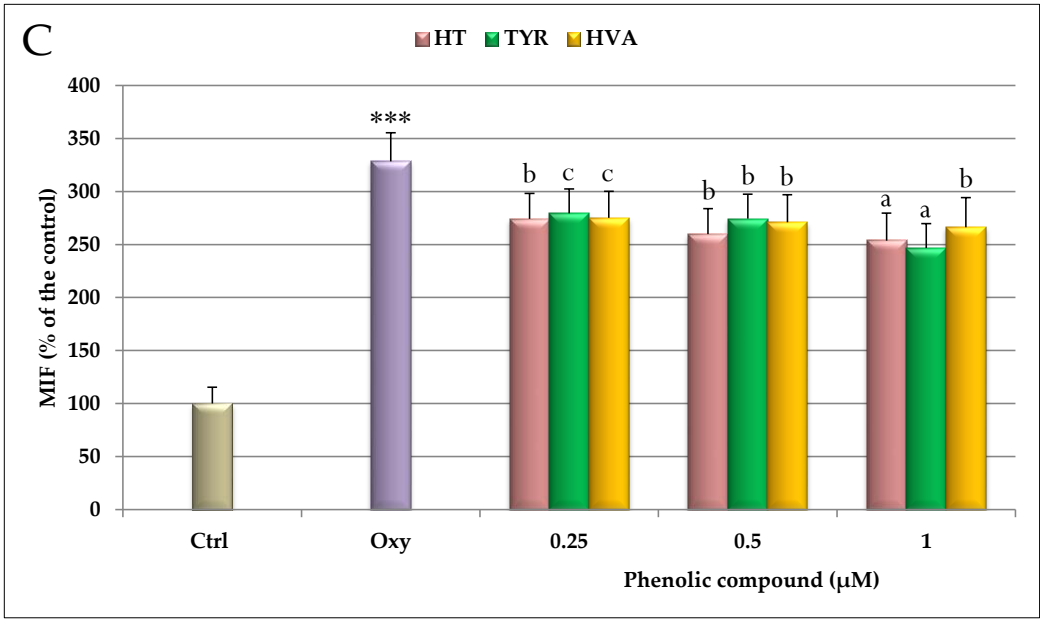
A preliminary screening was performed in order to evaluate which pro-inflammatory cytokines were produced in peripheral blood mononuclear cells treated for 24 h with the oxysterols mixture 20 μM.

The expression of four pro-inflammatory cytokines/chemokines was significantly different between the control and the samples treated with the oxysterols mixture in particular: IL-1β (Interleukin-1β), MCP-1 (Monocytes Chemo-attractant Protein-1), MIF (Macrophage migration inhibitory factor) and RANTES (Regulated on Activation, Normal T cell Expressed and Secreted).

Results of preliminary tests, was then confirmed by ELISA. Oxysterols mixture significantly increased the production of cytokines/chemokines MCP-1, IL-1β, MIF and RANTES in ex in-vivo cultured PBMCs after 24 h of incubation with the oxysterols mixture 20 μM. Hydroxytyrosol, tyrosol and homovanillic alcohol, at all tested concentrations significantly reduced the production of pro-inflammatory cytokines/chemokines induced by the oxysterols mixture (figure 27 A, B, C and D).


A
HT
TYR
HVA
MCP-1 (% of the control)
350
300
250
200
150
100
50
0
***
a
Ctrl
Oxy
0.25
0.5
1
Phenolic compound (µM)


B
HT
TYR
HVA
IL-1β (% of the control)
600
500
400
300
200
100
0
***
a
Ctrl
Oxy
0.25
0.5
1
Phenolic compound (µM)



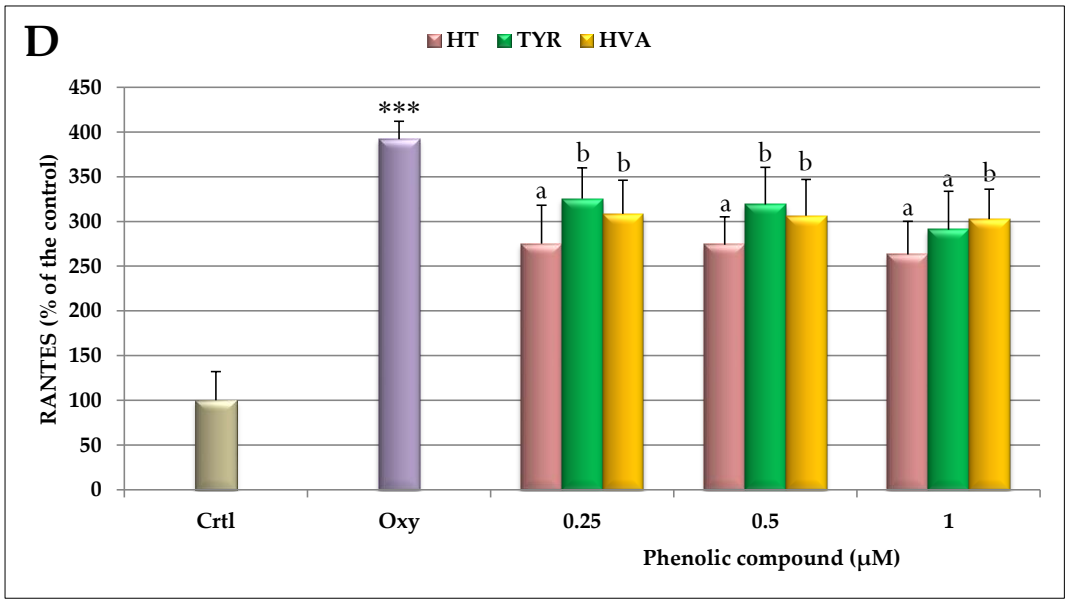


Figure 27: Effects of HT, TYR and HVA (0.25-0.5-1 µM) on MCP-1 (A), IL-1β (B), MIF (C) and RANTES (D) production in PBMCs treated with the oxysterols mixture 20µM for 24h.
*** = p<0.001 vs Ctrl; a = p < 0.001, b = p< 0.01, c = p< 0.05 vs oxy (n=6)
Control value: MCP-1 = 2400 pg/ml, IL-1β = 91 pg/ml, MIF = 770 pg/ml, RANTES = 2800 pg/ml

4.2.2 Intracellular ROS production

In PBMCs the level of cellular ROS production generated by oxysterols was also quantified, using the fluorescence probe DCFH-DA.

Before treatments, cells were incubated with 10 μM of DCFH-DA for 30 min at 37°C in the dark. As shown in Figure 28, cells were then treated with the oxysterols mixture 20 μM for different incubation times (15-180 min) and exhibited a significant increase in intracellular ROS production, starting after 15 minutes of incubation.

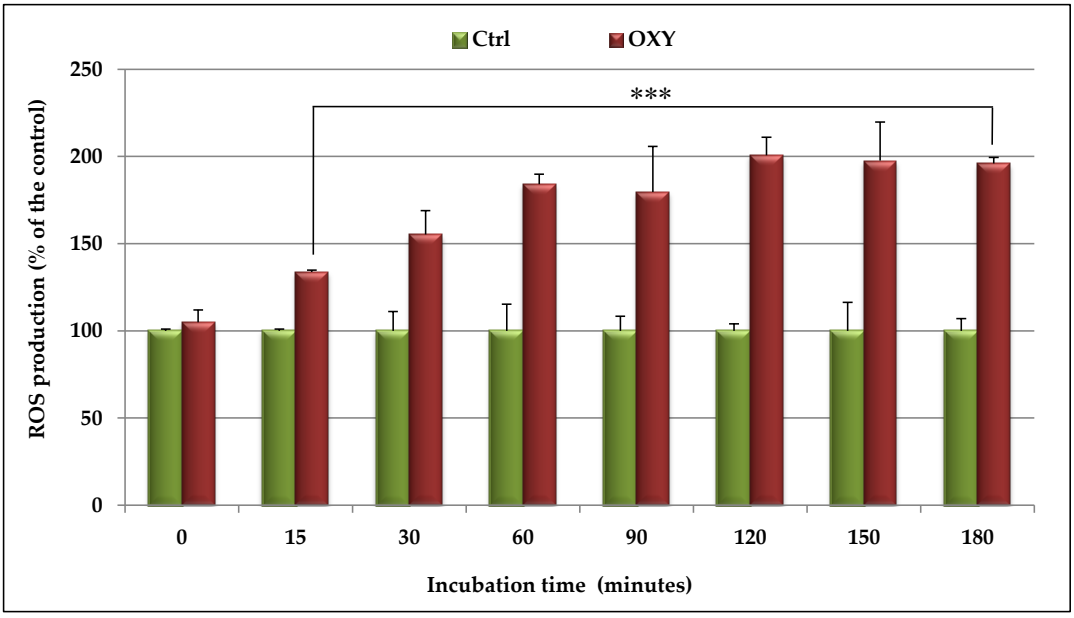


Figure 28: Intracellular ROS production (expressed as % of the control) in PBMCs treated with the oxysterols mixture 20 μM for different incubation times (15-180 min) using the fluorescence probe DFC-DA 10 μM for 30 minutes.
*** = $p<0.001$ vs ctrl (n=6)

As shown in Figure 29 pre-treatment of PBMCs for 30 min with pure phenolic compounds, hydroxytyrosol, tyrosol and homovanillic alcohol (0.25, 0.5, 1 μM) reduced intracellular ROS production induced by the oxysterols mixture 20 μM, significantly from the concentrations 0.5 μM.

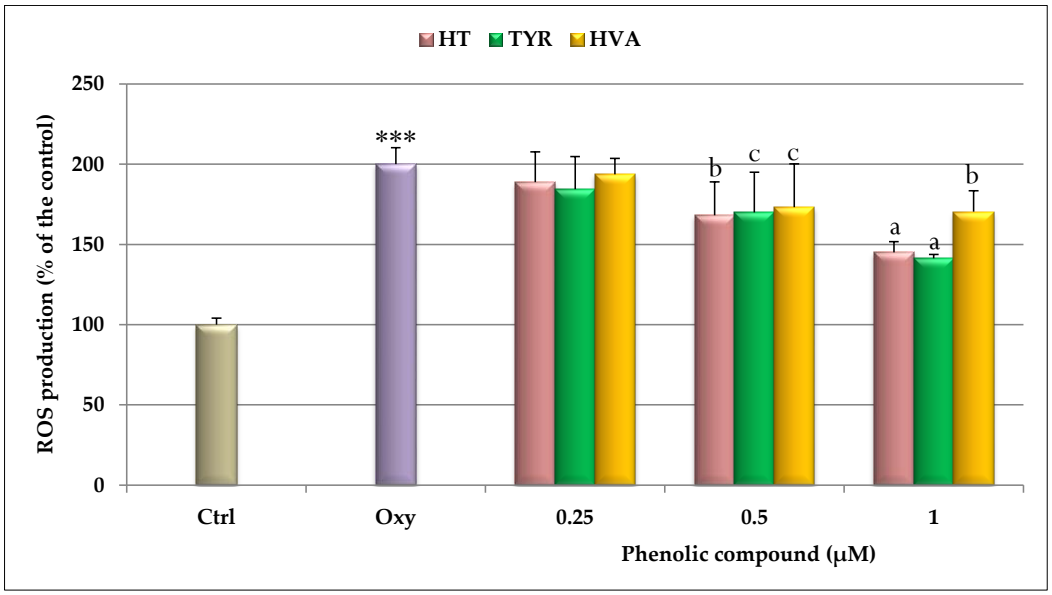


Figure 29: Effect of HT, TYR and HVA (0.25- 0.5- 1 µM) on intracellular ROS production in PBMCs treated with the oxysterols mixture 20 µM for 2 h.
*** = $p<0.001$ vs Ctrl; a = $p<0.001$, b = $p<0.01$, c = $p<0.05$ vs Oxy (n=6)

4.2.3 Modulation of intracellular signaling pathways

Intracellular signal transduction triggered by the oxysterols was investigated also in PBMCs.

The possible modulation of 2 mitogen-activated protein kinase (MAPK) cascade was analyzed in PBMCs treated with the oxysterols mixture 20 μM alone and in combination with pure phenolic compounds (HT, TYR and HVA). These kinases have been reported to be activated by various stress stimuli, and they have been also implicated in oxysterol-induced cytokine secretion and apoptosis.

4.2.3.1 JNK 1/2

Figure 30 shows the modulation of JNK 1/2 phosphorilation in PBMCs pre-treated for 30 min with HT, TYR and HVA (0.25-1 μM) and treated with the oxysterols mixture 20 μM for 3 h.

Oxysterols induced a remarkable increase in the level of the phosphorylated forms of JNK (p-JNK) in PBMCs after 3 h of incubation. Such increases were all prevented by the addition of phenolic compounds at all tested concentration, except for HVA, active only at 1 μM.

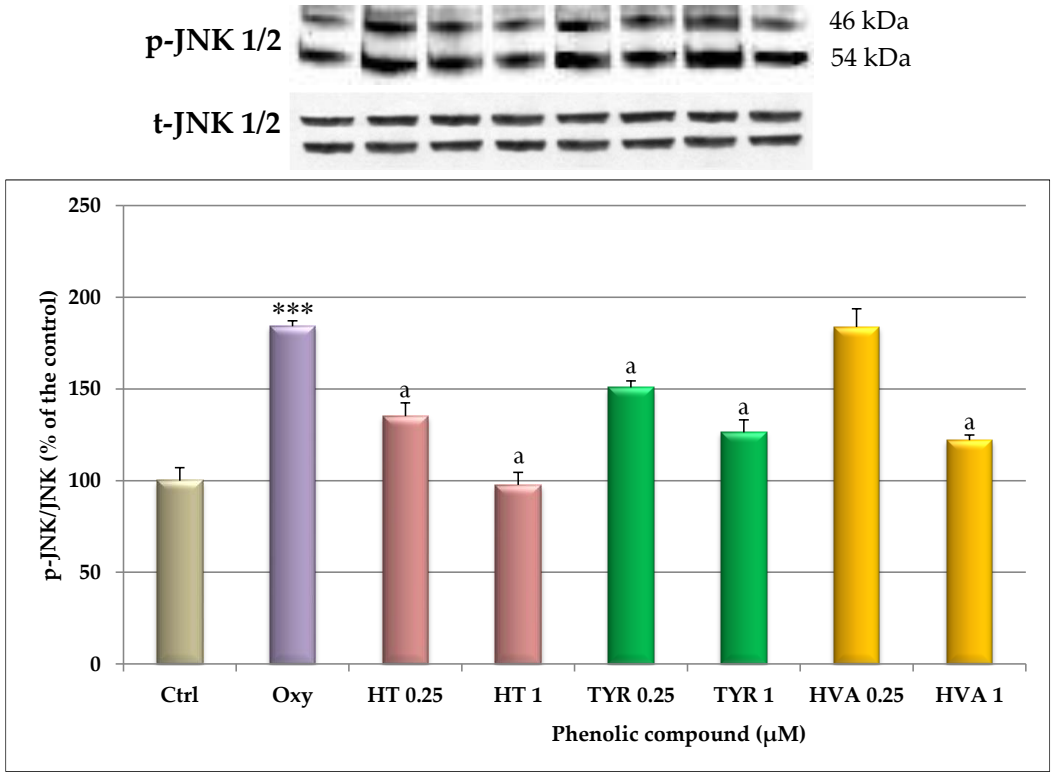


Figure 30: Modulation of JNK 1/2 in PBMCs pre-treated or not for 30 min with HT, TYR and HVA (0.25-1 µM) and incubated with the oxysterols mixture 20 µM for 3h.
***= p<0.001 vs Ctrl; a = p<0.001 vs Oxy (n=3)

4.2.3.2 P38

Changes also of p38 phosphorilation were analysed in PBMCs. As shown in Figure 31, PBMCs were pre-treated for 30 min with HT, TYR and HVA (0.25-1 μM) and treated with the oxysterols mixture 20 μM for 3 h. All phenolic compounds at the maximum tested concentration were able to counteract the increase of p38 phosphorilation induced by oxysterols in blood cells. HT was active also at 0.25 μM.

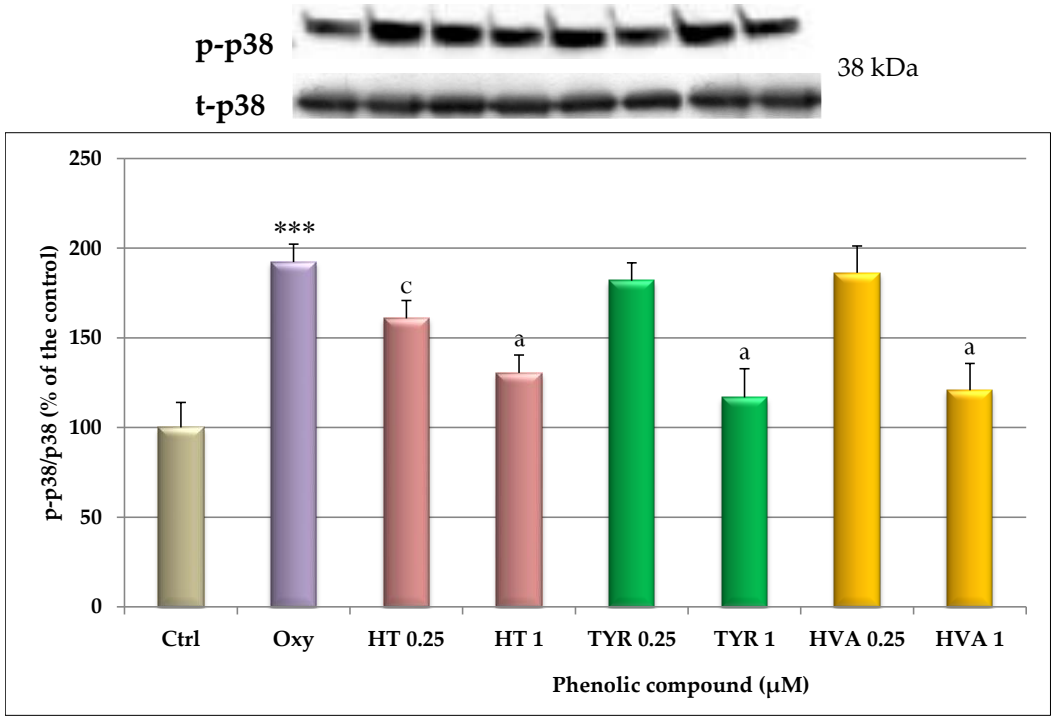


Figure 31: Modulation of p38 in PBMCs pre-treated or not for 30 min with HT, TYR and HVA (0.25-1 μM) and incubated with the oxysterols mixture 20 μM for 3h.
***= p<0.001 vs Ctrl; a = p<0.001, c = p<0.05 vs Oxy (n=3)

5. Discussion

Cholesterol oxidation products, termed oxysterols, may either originate endogenously, through enzymatic or non-enzymatic reactions, or may derive from the diet. As regards exogenous sources of oxysterols, foods containing cholesterol are susceptible to oxidation: oxidative reactions occur during food processing, mainly on exposure to heat treatment and during long-term storage **(Biasi et al. 2013a)**.

They are involved in physiological processes such as the regulation of cholesterol homeostasis, but it is well established that oxysterols have mostly detrimental biological activities. They provoke an imbalance of the ratio between oxidative and reductive biochemical reactions (oxidative stress) which acts on all organism level, from cell signaling to disease expression through up-regulation of inflammation, apoptosis and fibrosis **(Vejux et al. 2009, Otaegui-Arrazola et al. 2010)**.

Pathological accumulation of oxysterols may contribute in fact to the onset and especially to the development of major chronic diseases in which inflammation, but also oxidative damage and to a certain extent cell death, are hallmarks and primary mechanisms of progression. Indeed, certain oxysterols exercise strong pro-oxidant and pro-inflammatory effects at concentrations detectable in the lesions typical of atherosclerosis, neurodegenerative diseases, age-related macular degeneration, and other pathological conditions characterized by altered cholesterol uptake and/or metabolism **(Poli et al. 2013)**.

Only in the last few years, oxysterols have been shown to interfere with the homeostasis of the human digestive tract, demonstrating their involvement in the pathogenesis of human inflammatory bowel disease and colon rectal cancer **(Biasi et al. 2013b)**.

The gastrointestinal tract is constantly exposed to dietary oxidised food compounds produced during the reactions that occur during processing and storage of foods, such as lipid hydroperoxides and oxysterols that have been demonstrated to impact intestinal integrity, inducing a strong cytotoxic effect on

enterocytes, altering the redox state, triggering an inflammatory reaction and an oxidative damage **(Rezaie et al. 2007)**.

Experimental studies suggest that the ingestion of compounds with antioxidant action, such as phenolics, is able to counteract the oxidative stress and inflammatory response in the intestine and inhibit the onset of the main related diseases **(Romier et al. 2009)**.

Polyphenols are the most abundant antioxidants in the human diet and are found in many plant-derived products, i.e. fruits, vegetables, herbs and spices.

Several studies have shown that polyphenols are able to interact with biological systems and act as bioactive molecules, mainly due to their antioxidant activity, acting as scavengers of radical species, chelators of metal ions and modulating intracellular signaling pathways and gene expression **(Martin-Pelaez et al. 2013)**, and have also reported as having a strong anti-inflammatory effect both *in vitro* and *in vivo* **(Tripoli et al. 2005, Lopez-Miranda et al. 2010)**.

Extra virgin olive oil, the primary source of fat in the Mediterranean diet, is particularly important.

The association with consumption of extra virgin olive oil rich in phenolics with a lower incidence of various chronic degenerative diseases has been widely reported **(Cicerale et al. 2010)**.

The benefits of consuming olive oil have been known since antiquity and extensively reported and were traditionally attributed to its high content in oleic acid **(Quiles et al. 2006)**.

However, it is well established that these effects must be attributed to the phenolic fraction of olive oil with its antioxidant and anti-inflammatory activities.

The phenolic fraction of extra virgin olive oil contains different types of chemical compounds, mainly complex phenols, such as secoiridoids, derivatives of simple phenols hydroxytyrosol and tyrosol, which are the most biologically active **(Granados-Principal et al. 2010)**.

Dietary intake of olive oil polyphenols, in Mediterranean countries, has been estimated to be around 9 mg per day with a daily intake of 25–50 ml of olive oil; at least 1 mg is derived from free hydroxytyrosol and tyrosol, while 8 mg are related to their elenolic esters and to oleuropein and ligstroside aglycons **(de la Torre 2008)**.

Extra virgin olive oil complex polyphenols undergo a deep biotransformation in the gastro-intestinal level and some conjugated forms of the HT and TYR are hydrolysed in the stomach, increasing the amount of simple phenols that reach the intestine, where they undergo further metabolization. The phenolic compounds are poorly absorbed; don't reach blood concentrations of relief, most remains in the intestine, where especially the simple phenols can be present at particularly high concentrations (higher than μM) **(Corona et al. 2006)**. They might thus exert their health benefits by protecting intestinal mucosa from oxidative damage occurring during digestion, contributing to the enhancement of the antioxidant status as a whole **(Scalbert et al. 2000)**.

The first part of this research focuses on this context, with the aim of evaluate the antioxidant and anti-inflammatory activity of the phenolic fraction of two extra virgin olive oils obtained from two Sardinian cultivars, Bosana and Nera of Gonnos, against the harmful effect of a dietary oxysterols mixture in Caco-2 cells, an accepted model of human epithelial intestinal cell line.

Caco-2 cell line derives from a human colon adenocarcinoma, but cells undergo in culture a process of spontaneous differentiation that leads to the formation of a monolayer, expressing several morphological and functional characteristics of the mature normal enterocytes. Caco-2 cell monolayers represent a well-established model for the study of intestinal transport of nutrients and xenobiotics, and it is widely used in pharmacology and toxicology **(Artursson 1990, Baker et al. 1995, Meunier et al. 1995, Artursson et al. 2001)**.

Differentiated Caco-2 cells express microvilli, basolateral membranes separated by tight junctions, intestine-specific antioxidant enzymes such as superoxide

dismutase, glutathione peroxidase, glutathione reductase and catalase, and possess absorptive properties similar to those of normal intestine **(Rousset et al. 1985, Peters et al. 1989, Baker et al. 1992, Baker et al. 1993)**.

Cells were treated with a biologically compatible mixture of the most widely oxysterols present in cholesterol-rich foods: 7-ketocholesterol, 7α-hydroxycholesterol, 7β-hydroxycholestrol, 5α,6α-epoxycholesterol and 5β,6β-hydroxycholesterol **(Kanner 2007)**.

Most experimental studies reported to date have concerned individual cholesterol oxidation products, although these are always present in dietary lipids as a mixture **(Mascia et al. 2010)**. Their concentrations in foods have been quantified in the 10-100 μM range; the consumption of different foods containing oxysterols could lead to luminal concentrations of these products much higher than 100 μM **(Kanner 2007)**.

Depending on the experimental conditions used (incubation time/concentration of oxysterols mixture), oxysterols exerted different toxic effects on Caco-2 cells: at high concentration of mixture (150 μM), the alteration of the intracellular redox state and the induction of oxidative damage that led to cells death were observed; at lower concentrations oxysterols induced the activation of the redox-sensitive signaling pathways related to the cellular response to oxidative stress, cell survival/apoptosis and induction of the inflammatory response.

Changes in the cellular redox state were evaluated determining ROS generation, variation of intracellular GSH concentration and GPX activity.

The incubation with the oxysterols mixture induced a significant increase of intracellular ROS production after 60 minutes, as demonstrated by the reaction with dichlorofluorescein diacetate (DCFH-DA); a marked increase in fluorescence in the samples treated with the oxysterols, compared to control, was observed. This is in accordance with studies carried out by Biasi and colleagues using an equivalent oxysterols mixture **(Biasi et al. 2009, Biasi et al. 2013a)**.

The increase of ROS has led to a consequent decrease in the concentration of intracellular GSH, an antioxidant molecule abundant in the mucosal cells of the GI tract. Its primary function is to protect cells from oxidative stress detoxifying reactive oxygen metabolites of endogenous or exogenous origins **(Aw 2005)**.

Other studies carried out in different cell types showed the same results; it has been demonstrated that 7-KC and 7β-HC induced a significant decrease in GSH levels in PC12 cells and U937 **(Lizard et al. 1998, Lyons et al. 2001, O'Callaghan et al. 2001, O'Callaghan et al. 2002, Han et al. 2007)**.

However oxysterols, individually or in mixture, can exert opposite effects on the levels of intracellular GSH, depending on the experimental conditions used and the cell type **(O'Callaghan et al. 2001, O'Callaghan et al. 2002, O'Sullivan A et al. 2005)**.

Pre-treatment with both olive oil phenolic extracts significantly reduced the increase of ROS induced by oxysterols, at all tested concentrations.

Similar results were obtained in other studies; olive oil phenolics have been found to decrease ROS production (implicated in the development of a number of disease states) and elicit significant free-radical scavenging effects **(Goya et al. 2007, Paiva-Martins et al. 2009, Cioffi G et al. 2010)**. Phenolic compounds are believed to be capable of both direct reaction with ROS in the GI tract and of preventing their formation **(Halliwell et al. 2000)**.

For example, an olive oil phenolic fraction has been shown to protect colonic cells against injury induced by hydrogen peroxide **(Manna et al. 1997, Manna et al. 2002)**.

Moreover, in this study, a lower decrease of GSH was observed in cells pre-treated with phenolic extracts, confirming their ability to act as effective antioxidants.

Human studies have shown beneficial effects of olive oil phenolic compounds on markers of oxidative stress. Covas et al. **(Covas et al. 2006)** found that phenol-rich EVOO beneficially modulated the balance between gluthathione (GSH) and

gluthathione reductase (GSSG) and more recently olive oil phenolic compounds were found to increase GSH concentration in human blood **(Visioli et al. 2009)**

A significant increase in the activity of GPX was observed after 18 hours of treatment with oxysterols mixture; it represents a first line of defence against ingested oxidised lipids and is highly expressed in the intestinal tract; with other enzymes such as superoxide dismutase and catalase, it is part of the detoxification system of radical species responsible of the oxidative cell damage **(Piechota-Polanczyk et al. 2014)**.

Pre-treatment with the phenolic extracts decreased the enzymatic activity, probably because of the reduction of reactive species.

In our experimental system the phenolic extracts did not alter the basal activity of the enzyme. However many experimental studies demonstrated that cellular systems, treated with polyphenols, showed an increase of enzyme activities related to GSH, in particular of γGCS, Gred, GPX and GST **(Steele et al. 2000, Jeon et al. 2003, Molina et al. 2003, Scharf et al. 2003, Soto et al. 2003)**.

Effects of polyphenols on enzymatic activities related to GSH, in particular on GPX, were also demonstrated in *in vivo* studies, both in animal models and humans **(Rodrigo et al. 2002, Khan et al. 2004, Fito et al. 2005, Rodrigo et al. 2005)**, and a mechanism by which polyphenols exert these effects seems related to a direct action on enzymes gene expression **(Giovannini C. et al. 2006)**.

The treatment with the oxysterols mixture induced, in addition, a significant increase of MDA production after 24 h, indicating an advanced oxidative process of cellular macromolecules; this is in accordance with other studies that showed the ability of these compounds to produce MDA, for example in ECV-304 cells **(Wu et al. 2006)**, vascular smooth muscle cells **(Tang et al. 2005)** and liver mitochondria **(Liu et al. 2009)**.

Both phenolic extracts counteracted this increase, showing a significant protective activity at all tested concentrations; the activity of Bosana and Nera of Gonnos extracts was comparable.

Deiana et al. **(Deiana et al. 2010)** demonstrated that pre-treatment with olive oil simple phenols (HT, TYR and HVA) protected Caco-2 cells from oxidative damage induced by the lipid hydroperoxide TBH. Both HT and HVA, metabolite of HT in humans **(Manna et al. 2000, Tuck et al. 2002)** and detected as metabolite of HT also in Caco-2 cells **(Corona et al. 2006)**, inhibited the production of oxidation products, thus preserving the level of membrane lipids. It is noteworthy that TYR also exerted a protective action, limiting the oxidative damage, although this effect was achieved at a higher concentration with respect to the other phenols.

In spite of its relatively low antioxidant activity, TYR showed a stable protective effect against oxidized-LDL induced injury in Caco-2 cells; a study demonstrated that TYR inhibited membrane, modifications of cytoskeleton network, microtubular disorganization, loss of cell–cell and cell–substrate contacts, cell detachment and cell death **(Giovannini et al. 1999)**.

Similar results, regarding all aspects discussed above, were obtained in a study conducted in HpeG2 cells challenged with TBH; pre-treatment with hydroxytyrosol, the main phenolic acid present in olive oil phenolic fraction, significantly reduced the increase of ROS, GPX activity, and MDA, and the decrease of GSH induced by TBH **(Goya et al. 2007)**.

Intracellular ROS production by oxysterols may induce modification of cell proteins and alter various signaling pathways and gene expression **(Morrissey PA et al. 2006)**, leading to cell death.

A large number of *in vitro* studies has in fact described the potential pro-apoptotic effect of oxysterols in various cell systems **(Vejux et al. 2008)**.

In this study the induction of cell death by oxysterols was firstly analysed through Neutral red viability test, where a significant decrease of cell viability was observed from the concentration of 60 μM.

In order to clarify the molecular mechanisms involved in cell death, the induction of caspase 3 activation was evaluated. Caspase 3 is considered the most important

effector caspase and, as regards to oxysterol-induced apoptosis, it is the most widely studied.

Oxysterols induced a significant increase of caspase 3 activity in Caco-2 cells and this result agrees with many other studies.

Biasi and colleagues showed same results using an equivalent oxysterols mixture in intestinal cells **(Biasi et al. 2009, Mascia et al. 2010)**; authors showed that these oxysterols can exert their damaging effect in Caco-2 cells also when they are administered singly.

Studies from O'Sullivan and colleagues have shown that 25-HC, 5α,6α-EC, 5β,6β-EC or 7β-HC added individually to undifferentiated Caco-2 cells determined a reduction in cell viability assessed by the neutral red uptake assay **(O'Sullivan et al. 2003)**. The same research group further challenged undifferentiated Caco-2 cells for 24 h in the presence of 30 μM 5β,6β-EC, 7β-HC, or 7-KC, showing that cell viability, in terms of neutral red uptake, had decreased by 36, 11, and 17%, respectively **(Ryan et al. 2005)**.

However, the treatment of colon cancer cells with a diet-compatible mixture of oxysterols did not elicit the same responses than individual components added to the cells at the same concentrations at which they are present in the mixture. 60 μM oxysterols mixture showed a slight pro-apoptotic effect on human colon cancer Caco-2 cell line, evaluated in terms of caspase 3 and caspase 7 activation; conversely, 7α-HC, 7β-HC and 5α,6α-EC were identified to be able to induce a significant pro-apoptotic effect if added to cell culture singly; 7β-HC had stronger action than other compounds **(Biasi et al. 2013a)**.

This effect has been confirmed in other cell types treated with oxysterols, mainly involving 7β-HC or 7-KC: addition of 25-HC to 7β-HC or 7-KC significantly reduced the amount of oxysterol-induced apoptosis, which, on the contrary, was enhanced by 20-40 μM 7β-HC or 7-KC in human U937 pro-monocytic cells **(Aupeix et al. 1995, Larsson et al. 2006)**. Experimental studies on vascular cells reported that equimolar amounts of 7β-HC and 7-KC decreased the degree of

apoptosis caused by 7-KC alone in endothelial cells and in murine J774.A1 macrophages **(Biasi et al. 2004, Steffen et al. 2006)**. Conversely, synergistic cytotoxicity was found between 7-KC and 7β-HC, administered to monocytes in proportions similar to that found in atherosclerotic lesions **(Larsson et al. 2006)**.

In another study 30 μM of individual oxysterol components in the diet has shown that all 5α,6α-EC, 5β,6β-EC, 7-KC, and 7β-HC significantly reduced U937 viability and increased caspase activity and DNA fragmentation **(Kenny et al. 2012)**.

Pre-treatment with Bosana and Nera of Gonnos phenolic extracts protected cells against death, significantly increasing cell viability and reducing caspase 3 activity. A recent study showed similar results: Erol-Dayi Ö et al. investigated the protective effects of olive oil phenolic extract and one of its constituents, gallic acid (GA) against H_2O_2-induced oxidative stress and apoptotic cell death in HeLa cells, a model for human epithelial cells; both PE and GA were able to inhibit ROS production and the increase of caspase 9 induced by H_2O_2 **(Erol-Dayi et al. 2012)**.

Apoptosis is a multistep process, and protein kinases (MAPK, Akt/PKB) have been implicated in both the upstream induction phase of apoptosis and in the downstream execution stage, as direct targets for caspases **(Cross et al. 2000)**.

The modulation of these signals pathways was thus evaluated in the cells treated with the oxysterols mixture.

Akt has been well characterized as an antiapoptotic kinase that trasduces cellular survival signals in many cell types. The downstream actions of Akt appear to include phosphorylation of proteins involved in the apoptotic cascade and regulation of the expression of apoptotic proteins **(Cross et al. 2000, Maddika et al. 2007)**. Therefore, for apoptosis to occur, Akt must be inactivated early in the apoptotic process.

The results obtained in this study showed that exposure of Caco-2 cells for 30 min to the oxysterols mixture induced a significant decrease of Akt/PKB phoshorilation, confirming the induction of apoptotic process.

Other studies confirmed this effect, using oxysterols in mixture **(Biasi et al. 2009)**, and individually; in a murine macrophage-like cell line, 25-HC was found to induce apoptosis through degradation of Akt **(Rusinol et al. 2004)**. Berthier et al. **(Berthier et al. 2005)**, observed Akt inactivation during the initial steps of 7-KC-induced apoptosis in THP-1 cells. In support of these findings, two studies revealed that treatment of THP-1 macrophages with 7-KC coincides with decreased levels of Akt **(Palozza et al. 2007, Palozza et al. 2010)**. Moreover, 7β-HC induced apoptosis caused a decrease in Akt activation compared to untreated U937 cells **(Lordan et al. 2008)**.

MAPKs are a family of serine–threonine kinase enzymes which includes extracellular signal-regulated kinases ERK-1 and -2, JNK and p38. These proteins orchestrate the recruitment of gene transcription, protein biosynthesis, cell cycle control, apoptosis, and differentiation and allow cells to respond to oxidative stress and inflammatory stimuli **(Cross et al. 2000, Cardeno et al. 2014b)**.

Oxysterols mixture, in the experimental conditions of this study, was found to significantly reduce the phosphorilation of ERK 1/2, and to induce an increase of JNK and p38 phosphorilation in intestinal cells.

Activation of JNK and p38 is generally associated with the promotion of apoptosis, while ERK activity inhibits apoptosis **(Cross et al. 2000)**. Numerous *in vitro* studies have demonstrated modifications in ERK 1/2 activity following exposure to oxysterols **(Ares et al. 2000, Leonarduzzi et al. 2005, Prunet et al. 2006, Palozza et al. 2007)**. In C6 glioma cells, an early decrease in ERK 1/2 activity was found to correlate with the toxic effects of 7β-HC **(Adamczyk et al. 1998)**.

Following treatment with a different oxysterol, 7-KC, ERK 1/2 phosphorylation in THP-1 cells peaked at 2–3 h then declined toward basal levels after 12 h, while apoptosis increased significantly **(Berthier et al. 2005)**.

Other studies involving various cell lines have observed an increased ERK 1/2 activity immediately after cell challenge with oxysterols with no activation of JNK or p38 **(Ares et al. 2000, Berthier et al. 2005, Leonarduzzi et al. 2005)**. However,

this pattern of activity has not been consistently detected. Exposure to 7-KC for 24 h induced significant levels of apoptosis in THP-1 cells along with a remarkable increase in the level of the phosphorylated forms of ERK 1/2, JNK and p38 **(Palozza et al. 2007)**. Conversely, Yoon et al. **(Yoon et al. 2004)** showed that 22-HC activates ERK 1/2 and p38, but not JNK, in human cholangiocytes.

Interestingly, results obtained in this research showed that both phenolic extracts modulated all these pathways; the proapoptotic effects induced by oxysterols was completely reverted by the presence of olive oil phenolic extracts in cell medium; they were able to significantly reduce the decrease of Akt/PKB and ERK 1/2 phosphorilation, and to inhibit the phosphorilation of JNK and p38, suggesting an effective protection against oxysterols-induced cell death.

In the literature there aren't specific studies regarding the anti-apoptotic effets of olive oil phenolics in the GI tract, but their ability to counteract cell death was showed in other cellular systems. For example studies conducted by Deiana et al. demonstrated the ability of HT and its metabolite HVA to modulate Akt/PKB, ERK 1/2 and JNK phosphorilation, protecting kidney cells against cell death induced by H_2O_2 **(Deiana et al. 2008, Incani et al. 2010)**.

Several studies showed a strong modulatory effect of different classes of phenolic compounds on MAPKs, whose activity can be inhibited or increased depending on cell type and experimental conditions **(Santangelo et al. 2007)**.

MAPK are also involved in the early stages of the inflammatory response, as well as in the modulation of oxidative and apoptotic processes **(McCubrey et al. 2006)**.

The activation of MAPK pathway leads to the induction of a wide range of genes that contribute to the establishment of the immune response and inflammation, regulating the synthesis/activity of cytokines and chemokines, cell surface receptors, adhesion molecules and inflammatory enzymes. The inhibition of these intracellular signaling pathways, in any point of the cascade, allows a modulation of the inflammatory process **(Kaminska 2005)**.

JNK signaling pathway, classically linked to induction of apoptosis, plays an important role in the pro-atherogenic and pro-inflammatory effect of some oxysterols, in particular of 7-KC **(Pedruzzi et al. 2004)**.

It has also been shown that the pro-inflammatory action of some oxysterols (22-HC and α-triol) is mediated by the activation of p38 **(Yoon et al. 2004, Liao et al. 2009)**.

The activation of p38 is a key point of the inflammatory response in the intestine, since regulates the expression of several pro-inflammatory cytokines (IL-6, IL-8, COX-2, IL-1β, etc.), both in cells of the immune system in those structural of the intestinal wall, through modulation of transcription factors such as NF-kB, or the modulation of the stability and translation of specific mRNA **(Feng et al. 2001)**.

As demonstrated in *in vitro* models and humans **(Biasi et al. 2013b)** treatment with oxysterols induced the secretion of several cytokines such as IL-6 and IL-8, crucial mediators in sustaining the chronic intestinal inflammation.

In the experimental model of this study, oxysterols mixture showed the capability to induce the increase of IL-6 and IL-8; both phenolic extracts demonstrated a significant anti-inflammatory effect, inhibiting cytokines secretion.

Phenolic compounds derived from EVOO have been reported to have significant anti-inflammatory capacity. *In vivo* and *in vitro* research has suggested that the dietary intake of EVOO, containing significant concentrations of phenolics, may attenuate inflammatory responses in the body and therefore reduce the risk of chronic inflammatory disease development **(Corona et al. 2009, Khymenets et al. 2009, Konstantinidou et al. 2010)**.

Recent epidemiological studies have shown that habitual consumption of extra virgin olive oil is effective in the prevention of diverse types of digestive disorders such as inflammatory bowel disease. A recent study evaluated the protective effect of dietary extra virgin olive oil polyphenol extract supplementation in the inflammatory response associated to chronic colitis (DSS-induced) in rats; in EVOO+PE fed mice MCP-1, TNF-α, COX-2 and iNOS expression were

significantly reduced and in addition a down-regulation of JNK and p38 phosphorilation was observed, confirming the involvement of MAPK in the inflammatory process **(Sanchez-Fidalgo et al. 2013)**.

Another study investigated the ability of a phenolic extract from extra virgin olive oil to modulate the inflammatory response in intestinal epithelial cells (Caco-2). Phenolic extract prevented IL-8 expression and secretion induced by LPS and down-regulated the activation of p38 and JNK **(Muto et al. 2015)**.

Impellizzeri et al. **(Impellizzeri et al. 2011)** further found that the EVOO phenolic, oleuropein aglycone attenuates inflammatory responses in a carrageenan challenged mouse model of inflammation. Camargo et al. **(Camargo et al. 2010)** have recently shown that intake of an EVOO enriched-breakfast high in phenolics decreases the expression of several inflammatory genes including NF-KB and COX-2 among others.

Several studies in animals and humans have shown that dietary oxysterols, after digestion can be absorbed from the gut and transported into the circulation within chylomicrons and other lipoproteins **(Brown et al. 1999, Carpenter 2002)**. Furthermore, the presence of oxysterols in plasma can derive from the oxidation of endogenous cholesterol through enzymatic or spontaneous reactions **(Vejux et al. 2009)**.

Oxysterols have been found at increased levels in the plasma of hypercholesterolemic subjects and have been linked with the atherosclerotic process **(Leonarduzzi et al. 2012)**. Oxysterols in human plasma or serum may vary from about 1 μM (0.05% of total cholesterol) in healthy subjects to 20-30 μM (0.5-0.75% of total cholesterol) in diseased individuals **(Schroepfer 2000)**.

The intake of dietary antioxidant, as those present in extra virgin olive oil, could counteract the harmful effect of oxysterols, not only in the gastrointestinal tract even at systemic level.

In this context the second part of this study specifically focused on the protective effect of pure olive oil phenolics, which can be found in the blood stream after absorption (hydroxytyrosol, tyrosol, and homovanillic alcohol) against the pro-oxidant and pro-inflammatory activity of oxysterols in blood cells.

Peripheral blood mononuclear cells (PBMCs) separated from whole blood of healthy volunteers were used for this purpose; these are composed by lymphocytes, monocytes and macrophages, critical components in the immune system to fight inflammation and source of pro-inflammatory molecules.

PBMCs cells were treated with a biologically compatible oxysterols mixture composed by the most widely represented oxysterols in plasma of hypercholesterolemic subjects: 7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, cholesterol 5α,6α-epoxide, cholesterol 5β,6β-epoxide, cholestane-3β,5α,6β-triol and 25-hydroxycholesterol **(Biasi et al. 2004)** at pathologically relevant concentration (20 μM).

The first intent was to examine the ability of PBMCs to produce cytokines and chemokines in the presence of oxysterols, in particular MCP-1, IL-1β, MIF and RANTES, usually involved in pro-inflammatory processes; the oxysterols mixture was able to significantly increase the secretion of all cytokines/chemokines analysed.

Since it has been suggested that oxysterols may increase the levels of cytokines by modulating redox-sensitive pathways **(Palozza et al. 2011)**, the oxidative status of PBMCs treated with the oxysterols mixture was then measured.

An early increase (after 15 min) of intracellular ROS production was observed in PBMCs challenged with oxysterols compared to control.

At moderate concentrations, ROS may act as second messengers in signal transduction, by modulating redox-sensitive MAPKs; these kinases have been already reported to be activated by treatment with oxysterols and have been implicated in oxysterols-induced cytokine secretion **(Palozza et al. 2010)**.

In the experimental conditions of this research, the oxysterols mixture induced a significant increase of both JNK and p38 phosphorilation suggesting their involvement in cytokine secretion.

These results are in accordance with other experimental studies.

Up-regulation of MCP-1 has been reported in U937 promonocytic cells stimulated by a biologically relevant oxysterols mixture compatible with the same used in this study, and this activation was linked with a net increment of ERK 1/2 phosphorilation **(Leonarduzzi et al. 2005)**.

Furthermore, in human monocytic cells, 7β-HC and 25-HC, but also 7-KC to a lesser extent, are potent *in vitro* inducers of MCP-1, IL-1β, IL-8, TNF-α, and MIP-1β, as well as of other inflammatory molecules **(Prunet et al. 2006)**. The same study demonstrated that IL-8 secretion was associated with activation of the ERK 1/2 signaling pathway **(Prunet et al. 2006)**. The oxysterols 7-KC and 25-HC have also been observed to enhance IL-1β, IL-6, IL-8, and TNF-α mRNA and secretion levels, in a dose-dependent manner, although to different extents. These effects were associated with increased ROS production, and a net phosphorylation of MAPKs (ERK 1/2, JNK, p38) and NF-KB activation also occurred **(Palozza et al. 2011)**.

Up-regulation of IL-1β is another important event, because this cytokine increases the surface expression of endothelial adhesion molecules, by facilitating inflammatory cells attachment to the artery endothelium. Expression and synthesis of IL-1β were found to be stimulated by 25-HC in human macrophages, through the involvement of the LXR, as well as, but less strongly, by 27-HC **(Rosklint et al. 2002)**. IL-1β secretion was also markedly induced by 7β-HC, 7-KC, and 7α-HC in human promonocytic cells U937 and U4 **(Lizard et al. 1997, Lemaire-Ewing et al. 2005)** and in human umbilical vein endothelial cells (HUVECs) **(Lemaire et al. 1998)**.

Production of the proinflammatory cytokines TNF-α and IL-1β is also induced by 25-HC in adherent human peripheral blood mononuclear leukocytes, through phosphorylation of p38 MAPK **(Feng et al. 2001)**.

In the literature there isn't any specific correlation between oxysterols and RANTES and MIF secretion; but the increased RANTES and MIF expressions have been associated with a wide range of inflammatory disorders and pathologies, such as inflammatory bowel disease and atherosclerosis where oxysterols exert a pivotal role.

RANTES and MIF are cytokines with chemokine-like function and critical mediators of the host immune and inflammatory response; they are thought to act by recruiting and promoting leukocytes infiltration to sites of inflammation **(Appay et al. 2001, Asare et al. 2013)**.

In this study it was demonstrated that all phenolic compounds HT, TYR and HVA at all tested concentrations, were able to inhibit oxysterol-induced pro-inflammatory cytokines production in immune cells.

According to the hypotesis that pro-inflammatory cytokines release may be induced by changes in intracellular redox status, it was observed that, in human PBMCs treated with the oxysterols mixture, simple phenols were able to inhibit ROS production significantly from the concentration of 0.5 μM as well as to suppress both redox-based MAPK phosphorylation (JNK, p38).

In the literature there are several studies that confirmed the anti-inflammatory activity of olive oil phenolics in different districts such as in blood cells.

Differential anti-inflammatory effects of phenolic compounds from extra virgin olive oil were identified in human whole blood cultures: oleuropein glycoside and caffeic acid decreased the concentration of IL-1β and kaempferol decreased the concentration of PGE2 induced by LPS **(Miles et al. 2005)** .

A recent study reported similar results in macrophages where oil phenolic extracts exerted their protective effects against LPS-induced oxidative stress and inflammatory responses **(Cardeno et al. 2014a, Cardeno et al. 2014b)**. The olive oil

PE significantly decreased NO (nitric oxide) and ROS production and in addition significant down regulated iNOS, COX-2 and mPGES1 protein expression, reduced MAPK (JNK, p38) phosphorilation and prevented the nuclear NF-KB traslocation.

HT inhibits iNOS and COX-2 expression in LPS-stimulated J774 cells at the transcriptional level by preventing the activation of NF-KB, STAT-1α and IRF-1 **(Maiuri et al. 2005)** and TYR prevented RAW 264.7 macrophages activation induced by gliadin and INFγ **(De Stefano et al. 2007)**.

A recent study conducted by Palozza et al. **(Palozza et al. 2011)** investigated the ability of lycopene, a strong antioxidant compound present in tomato, to prevent oxysterols induced pro-inflammatory cytokine cascade in human macrophages.

Lycopene prevented oxysterol-induced increase in pro-inflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α) secretion and expression, such an effect was accompanied by an inhibition of oxysterols-induced ROS production and MAPK (ERK 1/2, JNK and p38).

Olive oil phenolics analysed showed similar capabilities against the harmful effects of the oxysterols mixture in ex-vivo blood cells.

Further studies are needed to clarify the mechanism by which the phenolic compounds exert their protective action; however, data obtained in these experimental systems, suggested that these compounds act primarily by counteracting the initial stages of the pro-oxidant and pro-inflammatory effects of oxysterols, inhibiting the formation of ROS and then all subsequent cascading effects.

In conclusion data reported in this study confirmed the ability of oxysterols, of both endogenous and exogenous origin, to play important roles in different districts, involving cytotoxic, pro-oxidant and pro-inflammatory processes, in particular in the gastro-intestinal tract, constantly exposed to dietary oxidised lipids and at systemic level where these compounds can be found after absorption. The olive oil phenolics exerted a protective effect in both systems.

The Bosana and Nera of Gonnos extracts as a whole may contribute to preserve the integrity of intestinal mucosa, scavenging free radicals, preventing lipids peroxidation, enhancing endogenous defences, preventing cell death and cytokines production through the modulation of different protein kinases.

These results suggest a possible preventive effect of olive oil phenolic fraction on intestinal diseases related to oxidative stress and inflammation.

Both extracts demonstrated the same effectiveness; however Bosana extract has been shown to contain a higher amount of phenolic contents in particular HT, TYR and their secoiridoids derivatives. The high concentration of phenolic in an extract is not crucial for its biological activity, but depends on the molecular structures of the individual phenolic compounds present and the possible synergy or antagonism between the different classes of compounds.

Olive oil simple phenols, derived from metabolism and absorption of whole extracts, exerted their protection also at systemic level specifically reducing ROS production and cytokines secretion, modulating MAPK; HT, TYR and HVA showed a comparable significant action.

These findings may indicate antioxidant and anti-inflammatory properties of absorbed phenolics, which may be therapeutically useful in controlling chronic immune and/or inflammatory processes, such as atherosclerosis or neurodegerative diseases.

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